SYRIAN HAMSTERS AS CLINICALLY RELEVANT MODELS FOR MOOD DISORDERS

A Dissertation

by

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ABSTRACT

Anxiety and depressive disorders are the two most common types of mood disorders in the United States. These disorders are more likely to affect women than men. Animal models do not cover key aspects of mood disorders well, including sex biases, links between metabolism, HPA activity, social status and anxiety and depression as well as paradoxical effects of antidepressants on some adolescents. It is reported here that two novel tests, the anxiety feeding/exploration conflict (AFEC) test and the reward/investigational preference (RIP) test, are suitable for measuring anxiety-like and depression-like behavior in Syrian hamsters (*Mesocricetus auratus*). Furthermore, biometrics including body weight, food intake, indirect calorimetry, plasma assays and tissue weights measured metabolism. Gene expression and electrophysiology were used to measure brain function. Ph.armacological interventions manipulated behavior and metabolism. Animals were manipulated through social housing (SH, 2 or more animals per cage) and social separation (SS, one animal per cage) and pharmacology to model mood disorders in humans.

Relative to SH, SS hamsters show a combination of increased metabolic rate, hypophagia, decreased adiposity, decreased lean mass, low HPA activity and increased anxiety-like and depression-like behaviors with a profound female sex bias. Relative to adult female Syrian hamsters, adolescent female Syrian hamsters show aberrant responses to the antidepressant fluoxetine including increased anxiety-like behavior, increased depression-like behavior and anxiety-like and depression-like behavior that do not improve over the course of the experiment. In addition, there are divergent responses of gamma-amino butyric acid (GABA) miniature inhibitory chloride currents and divergent GABA-related gene expression. Thus, the Syrian hamster model shows advantages over related models in rats and mice for some aspects of modeling anxiety and depression.

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CONTRIBUTORS AND FUNDING SOURCES

Contributors

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Plasma corticosteroid and ACTH data analyzed in section 3 were provided by Caurnel Morgan. Part of the body weight and food intake data on adults in section 3 were provided by Caurnel Morgan and reproduced and added to by the work here. mIPSC data on hamsters treated with fluoxetine or vehicle in section 4 was collected and analyzed by Dustin DuBois and Annette Fincher in the laboratory of Gerald Frye.

All other work in this dissertation was completed by the student independently with the consultation of committee members (especially Caurnel Morgan).

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NOMENCLATURE

Gene Abbreviations: *Bdnf* – Brain-Derived Neurotropic Factor; *Creb1* – Cyclic AMP Response Element Binding protein; *Crf* – Corticotropin Releasing Factor; *FosB* – Fos-related Antigen B; *ΔFosB* – delta Fos-related Antigen B; *Gabra*(n) – GABA-A receptor alpha subunit n; *Gabrb*(n) – GABA-A receptor beta subunit n; *Gabrg*(n) – GABA-A receptor gamma subunit n; *Gabrd* – GABA-A receptor delta subunit; *5HT1a* – Serotonin Receptor 1a; *Il6* – Interleukin 6; *Tlr4* – Toll-like Receptor 4

AFEC – Anxiety Feeding/Exploratory Conflict test

B – Corticosterone, Compound B

Dia – Diazepam

Dmi – Desipramine

DNQX – 6,7-dinitroquinoxaline-2,3-dione

Dx-p – Dexamethasone Phosphate

F – Cortisol, Compound F

Flu – Fluoxetine

GABA – γ-Amino Butyric Acid

GABA-A – GABA Type A receptors; ionotropic GABA receptor

HPA – Hypothalamic-Pituitary-Adrenal axis

mEPSC – Miniature Excitatory Post Synaptic Current

mIPSC – Miniature Inhibitory Post Synaptic Current

M-MLV – Moloney Murine Leukemia Virus reverse transcriptase

NAc – Nucleus Accumbens

Nal – Naltrexone

NSF – Novelty Suppressed Feeding test

PCR – Polymerase Chain Reaction

Pro – Propranolol

RIP – Reward/Investigational Preference test

SH – Social Housing

SS – Social Separation

SSRI – Selective Serotonin Reuptake Inhibitor

Trac – Tracazolate

TTX – Tetrodotoxin

Veh – Vehicle for drug injection

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INTRODUCTION

Anxiety disorders and depressive disorders are the two most common types of mood disorders in the United States. Each year an estimated 12% and 18% of Americans over 12 are affected by depression (Pratt and Brody 2012) and anxiety (Kessler *et al* 2005), respectively. Depression and anxiety are comorbid and are twice as likely to affect women as men (Grant *et al* 2009). The need for females in animal models of anxiety and depression has been recognized for many years because of the higher incidence of these disorders in women than men. Furthermore, there are known differences in how males and female rodents react to stress and social context (reviewed in Donner and Lowry 2013, Palanza 2001), sex differences in modulatory neurotransmitter targets of antidepressant drugs and differences in how male and female animals react to behavior tests (reviewed in Donner and Lowry 2013). Animal behavior models incorporating females remain scarce.

The United States Food and Drug Administration issued a black box warning on the use of antidepressants in people under the age of 25 after meta-analyses of clinical trials have found a link between initial treatment of adolescents and young adults with antidepressants worsening on symptoms for anxiety (Bridge *et al* 2007) depression (Bridge *et al* 2007, Cusin *et al* 2007) and suicidality (Stone *et al* 2009, Bridge *et al* 2007, Hammad 2006, Jick *et al* 2004). Antidepressants only show modest therapeutic efficacy in adolescents (Bridge *et al* 2007, Whittington *et al* 2004) or are statistically indistinguishable from placebo in one meta-analysis (Mann *et al* 2006). Due to the limits on human experimentation, an animal model of paradoxical effects of adolescent antidepressant treatment would be useful in understanding mechanisms of these effects and adjusting therapies for younger people. So far, attempts to model paradoxical effects in adolescents with continuous antidepressant treatment have only had limited success requiring surgical implants for drug administration (West at al 2010) or antidepressant discontinuation (Homberg *et al* 2011). Surgery and discontinuation are confounds

not resembling antidepressant treatment in humans. Other researchers have failed to produce paradoxical antidepressants effects in adolescent rat and mouse models (Karanges *et al* 2011, Iñiguez *et al* 2010, Oh *et al* 2009, de Jong *et al* 2006).

Anxiolytic and antidepressant-sensitive tests for anxiety-like (Merali *et al* 2003, File *et al* 1993, Misslin *et al* 1989) and depression-like (Wilner *et al* 1987, Steru *et al* 1985, Porsolt *et al* 1979, Porsolt *et al* 1977) behaviors have been developed to model mood disorders in animals. Learning during exploration-based behavior tests alters responses to anxiolytic treatments making them insensitive in the absence of a specific threat such as predator odor (reviewed in File 1993). Sucrose preference test measures depression-like behavior (decreased hedonic drive) and responds to chronic antidepressant treatment, but it requires a labor-intensive schedule of chronic stress (Kubera *et al* 2013, Muscat *et al* 1992, Willner *et al* 1987) which makes repetition difficult. Progressive ratio measures hedonic drive, but it can be labor and time intensive, is unreliable in measuring effects of drugs or stressors with short duration effects and subjects becoming sated with or developing tolerance to a reward (reviewed in Stafford *et al* 1998).. Tests that remain sensitive to anxiolytics and antidepressants with repetition would be useful in measuring time courses of treatments that alter emotional status.

The aforementioned studies point to 3 gaps in animal models of anxiety and depression. First, more animal models of anxiety and depression with female sex bias would be useful in elucidating neurological underpinnings of sex bias in anxiety and depression in humans. Second, animal models showing paradoxical effects of antidepressants in adolescents but not adults without the confounds of surgery and discontinuation would help elucidate the neurobiological underpinnings of paradoxical responses of antidepressants in adolescents. Finally, low labor and time intensity, repeatable tests for anxiety-like and depression-like behaviors would aid in the study of anxiety and depression.

The following studies address these knowledge gaps using Syrian hamsters (*Mesocricetus auratus*). Section 2 validates two new behavior tests: anxiety feeding/exploration conflict test (AFEC) and reward/investigational preference (RIP) test. AFEC and RIP tests are low stress, low labor intensity, can be performed together, distinguish anxiety-like and depression-like behavior, respectively, from appetitive behavior and can be repeated on the same hamsters. Section 3 presents an experimental paradigm in hamsters linking moving animals from social housing (SH) to social separation (SS) with female-biased voluntary food restriction, increased metabolic rate, decreased HPA activity, decreased adiposity and lean mass and increased anxiety-like and depression-like behavior. Section 4 shows adolescent female hamsters but not adult females have paradoxical increased anxiety-like and depression-like responses to fluoxetine. The study focuses on GABA-A signaling and subunit plasticity in the nucleus accumbens (NAc) as one potential mediator of the paradoxical effects.

VALIDATION OF ANXIETY FEEDING/EXPLORATION CONFLICT (AFEC) TEST AND REWARD/INVESTIGATIONAL PREFERENCE (RIP) TEST FOR MEASURING ANXIETY-LIKE AND HEDONIC DRIVE-LIKE (DEPRESSION-LIKE) BEHAVIOR IN SYRIAN HAMSTERS (*Mesocricetus auratus*)*

Summary

Food reward based tests are used to assess anxiety using latency to feed and depression using effort or amount consumed as an index of hedonic drive. It is often unclear whether consummatory drive, appetite or emotional status drive animal performance in these. Furthermore, results from repeated tests on the same animal are often not comparable and tests are often stressful for animals and/or labor intensive for researchers. Anxiety feeding/exploration conflict (AFEC) test assesses anxiety-like behavior and reward/investigational preference (RIP) test assesses depression-like (reduced hedonic drive) behavior in Syrian hamsters. Both prevent the hamster appetitive behavior of cheek pouching. Hyperphagia is not associated with improved performance in either test. Both tests are no more stressful than a cage change, are low labor intensity, can be performed together and can be performed repeatedly with results comparable from test to test. Both tests use highly palatable food reward to produce two metrics of assessed behavior and have internal controls for motoric and non-specific effects. AFEC produces feed latency and feed latency ratio (test cage:home cage latency) and home cage feed latency as a control. RIP produces reward investigation time (time spent investigating a cassette containing an inaccessible highly palatable food reward), reward investigation preference (percent of total investigation time spent investigating reward cassette) and blank investigation time (an empty "blank" cassette). AFEC performance is improved by anxiolytic drugs, worsened

*Part of the data in this section reprinted with permission from A modified anxious behavior test for hamsters. Shannonhouse *et al*., 2014 J Neurosci Methods. 221:62-9.

by an anxiogenic drug and insensitive to drugs unknown to affect anxiety. RIP performance is improved by chronic antidepressant treatment and is insensitive to short term antidepressant treatment and drugs that are not known to affect depression. Results show AFEC and RIP are valid for repeat measures of anxiety-like and depression-like behavior in hamsters.

Introduction

Anxiety disorders and depressive disorders are the two most common types of mood disorders in the United States. Each year an estimated 12% and 18% of Americans over 12 are affected by depression (Pratt and Brody 2012) and anxiety (Kessler *et al* 2005), respectively. Depression and anxiety are comorbid and are twice as likely to affect women as men (Grant *et al* 2009). The annual economic costs of depression in the United States in 2010 was estimated to be $80 billion from Major Depressive Disorder alone and over $210 billion when comorbid disorders are considered (Greenberg *et al* 2015).

The objective of this section is to validate two novel tests for emotional behavior in Syrian hamsters (*Mesocricetus auratus*): anxiety feeding/exploration conflict (AFEC) test and reward/investigational preference test (RIP). Advantages of the tests include distinguishing appetitive and consummatory confounds away from anxiety-like and motivational and decisional hedonic drive, respectively, that they can be performed together and that they do not require a labor intensive schedule of chronic stress.

Anxiolytic and antidepressant-sensitive tests for anxiety-like (Merali *et al* 2003, File *et al* 1993, Misslin *et al* 1989) and depression-like (Wilner *et al* 1987, Steru *et al* 1985, Porsolt *et al* 1979, Porsolt *et al* 1977) behaviors have been developed to model mood disorders in animals. Learning during behavior tests alters responses to anxiolytic treatments (reviewed in File 1993). Chlordiazepoxide does not reduce open-arm time in rats using elevated plus maze subsequent to the first trial under normal conditions, but they do reduce phobic responses to predator odor (File and Zangrossi 1993). Similarly,

chlordiazepoxide's anxiolytic effects were more difficult to detect in the light/dark box test in mice by one prior exposure to the test apparatus. However, novelty-suppressed feeding (NSF) on highly palatable foods in mice showed anxiolytics reduced feed latency relative to controls over several trials (Merali *et al* 2003). This shows NSF is a valid tool to measure changes in anxious state over time.

Syrian hamsters (*Mesocricetus auratus*) do not increase food intake after periods of food deprivation (Schneider *et al* 2000, Borer 1985). Unlike rats and mice, appetitive drive in hamsters manifests in increased cheek pouching but not hyperphagia (Wong and McBride 1993, Arbour and Wilkie 1988). Therefore, preventing cheek pouching in a food reward based test is a way to distinguish anxiety-like behavior from both appetitive and consummatory behavior.

Hedonic drive is an important component of depression (Sharpley and Bitsika 2013). Sucrose preference test measures hedonic drive and responds to chronic antidepressant treatment, but it requires a labor-intensive schedule of chronic stress (Kubera *et al* 2013, Muscat *et al* 1992, Willner *et al* 1987). A similar, less labor-intensive test would be useful. Hedonic drive can be separated into appetitive (reward anticipation), motivational (wanting), consummatory (liking) and decisional (use of information on reward to make decisions) components (Yoshikawa *et al* 2013, Treadway *et al* 2012, Smith *et al* 2011, Treadway and Zald 2011, Barr and Phillips 1999, Barr and Phillips 1998). A food reward test preventing cheek pouching and eating would allow a test of hedonic drive to isolate motivational and decisional components.

This section shows AFEC and RIP are valid tests in Syrian hamsters for anxiety-like behavior and hedonic drive, respectively, they remain valid after repetition and they do not require any labor intensive schedule of stress.

Materials and Methods

Animals

Syrian hamsters (*Mesocricetus auratus*) of the Lake View Gorge strain (Charles River, Kingston, NY) were purchased for use at age 8 weeks or were bred in the Kleberg Laboratory Animal Facility at Texas A&M University. Animals were kept on a 14h:10h light:dark schedule (lights on at 0600h) at 23 ± 3˚C. LabDiet 5001 (Purina, Richmond, IN) and water were provided ad libitum. Food intake was monitored by weighing food from the food hopper, the bedding and cheek pouches to the nearest 0.1g. Bedding was Sani-Chips (Murphy's Products, Monteville, NJ). Each cage was supplied with Nestlets (Ancare, Bellmore, NY). Males were single housed from age 10 weeks in the diazepam, propranolol and desipramine experiments. Males were group housed in the fluoxetine experiments. Females were single housed from age 10 weeks in the propranolol and all food intake experiments. Females were group housed in the diazepam and fluoxetine experiments. Procedures used were approved by the Institutional Animal Care and Use Committee.

Anxiety-related feeding/exploration conflict test

Latency to feed on a highly palatable food (Merali *et al* 2003) was timed. To prevent cheek pouching, instead of placing the food on the floor of the cage, graham cracker (Nabisco, East Hanover, NJ) was presented overhead in a spring-loaded clamp attached to a bar supported by a ring stand. To discourage climbing out of the cage, the cracker was lower through a hole in the center of a polycarbonate cover. The cages were polycarbonate and placed on a white surface in a test area lit to ~1000 lux. The setup prevented cheek pouching which is an appetitive, but not anxious, behavior (Chester *et al* 2006). Approach latency in the test and home cages were measured to assess non-anxiety related factors (appetitive, consummatory, olfactory and motoric) under aversive and non-aversive conditions, respectively. Test cage:home cage feed and approach latency ratios are an alternate, normalized measure to control for non-anxiety-

related factors. Tests were conducted between 1000h and 1600h in a dedicated behavior room because peak anxiety for hamsters has been reported at 1300h (Yannielli *et al* 1996). Behavior scorers were blind to treatments. Food was removed about 1.5 hours before testing. Since hamsters eat approximately every 2 hours (Oklejewicz *et al* 2001, Borer *et al* 1985), they had all missed one meal at the time of the test. Animals were given about 30mg/day graham cracker for 7 days before the first test.

Reward/investigational preference test (RIP test)

RIP is based on the principle that animals experiencing anhedonia will spend less time and effort investigating something rewarding relative to non-rewarding control similar to the sucrose preference test (Willner *et al* 1987) or the female encounter test (Ago *et al* 2015). Animals were allowed at least 10 minutes to acclimate to the test cage. Reward (graham cracker) and blank (empty) cassettes were placed in an overhead food hopper or lowered into the cage suspended by spring-loaded clamps attached to a bar supported by a ring stand. Cassettes were clear plastic containers of MiniDV camcorder tape. Time spent investigating each cassette was measured with a stopwatch. Increases in reward investigation time and reward investigation preference (100% * reward investigation time / [reward + blank investigation time]) were used as indices of hedonic drive.

Drugs

Diazepam (0 or 1mg/kg, i.p.) was dissolved in ethanol or 0.9% saline. Propranolol (0, 2mg/kg, 4mg/kg or 10mg/kg, i.p.) was dissolved in 0.9% saline. Desipramine (20mg/kg/day) and fluoxetine (10mg/kg/day) were dissolved in drinking water at 0.34 and 0.17mg/mL, respectively, assuming water intake of 8mL/day (Lee *et al* 2001, Richardson *et al* 1992). Additionally, for injection on Day 1, desipramine (20mg/kg, i.p.) and fluoxetine (10mg/kg, i.p.), respectively, were dissolved in 0.9% saline.

Food intake

Female hamsters were individually housed (socially separated) for 5 weeks (propranolol experiment) or 4 weeks (desipramine and fluoxetine experiments). Animals were kept in their home cage for the entire experiment. In the propranolol (Pro) experiment, food was removed and weighed at t = 0, drug injections occurred at t = 2 hours, food was returned at t = 2.5 hours and remaining food was weighed at t = 4.5 hours. Number of animals for veh (2mg/kg Pro), pro (2mg/kg), veh (10mg/kg) and pro (10mg/kg) were 9, 9, 14 and 5 animals, respectively. For chronic antidepressant in the drinking water treatment, animals had vehicle (veh, drinking water), desipramine (20mg/kg/day, po) or fluoxetine (10mg/kg/day, po) for 3 weeks. On the last day of weeks 1, 2 and 3, overnight food intake was measured. Number of nights of food intake measured for veh, flu and dmi were 9, 21 and 21, respectively.

Statistical analysis

Two-way ANOVA (Fig 1-1 and 1-2) and 2-way RM ANOVA (Fig 1-3, 1-4, 1-5) were performed using Prism 5.04 and InStat 3.00 (GraphPad, San Diego, CA).

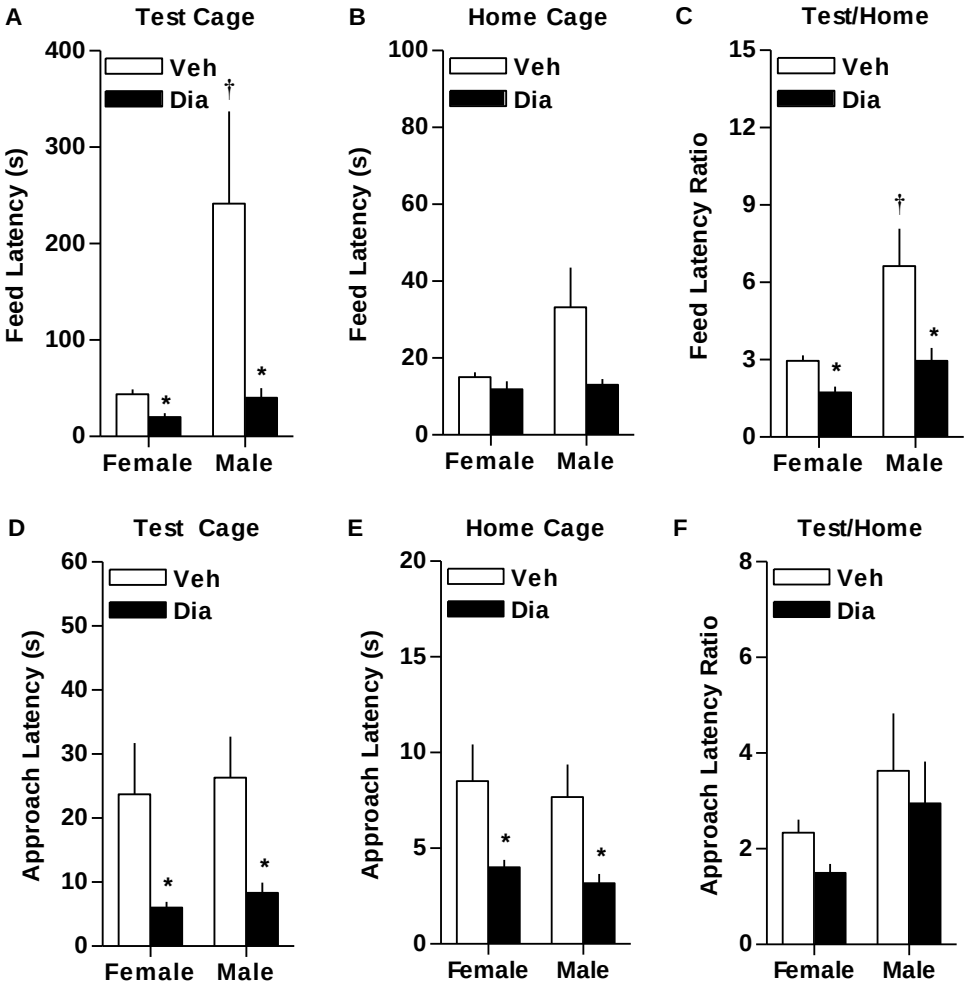

Figure 1-1: **Diazepam reduces feed latency in AFEC. Male and female hamsters** (n = 5-6) were injected with diazepam (0 or 1mg/kg) and assessed in the AFEC test after 30 minutes. (A) Relative to vehicle (Veh), diazepam (Dia) treatment reduced feed latency in males and females. Relative to vehicle treated females, vehicle treated males showed higher feed latency. (B) There were no statistically detectable differences in home cage feed latency between Veh and Dia treatment in males or females. Males and females were not statistically distinguishable. (C) Relative to Veh, Dia treatment reduced test cage:home cage feed latency ratio. Relative to Veh treated females, Veh treated males showed higher test cage:home cage feed latency ratio. (D) Relative to Veh, Dia treatment reduced approach latency in males and females. Males and females were not statistically distinguishable. (E) Relative to Veh, Dia treatment reduced home cage approach latency in males and females. Males and females were not statistically distinguishable. (F) This treatment did not alter test cage:home cage approach latency ratio. *p<0.05 between Veh and drug, †p<0.05 between sexes. (from Shannonhouse *et al* 2014b)

Results

Effects of known anxiolytics on AFEC

The purpose of these experiments was to see if anxiolytics would reduce feed latency in AFEC. Relative to vehicle (Veh), diazepam (Dia) decreased feed latency and test cage:home cage feed latency ratio in males and females. There were no statistically detectable differences in home cage feed latency (Fig 1-1A-C). Relative to Veh, Dia decreased approach latency in both the test cage and the home cage. There were no statistically detectable differences in normalized approach latency (test cage:home cage approach latency) (Fig 1-1D-F). Relative to Veh, propranolol (Pro) decreased feed latency and normalized feed latency (test cage:home cage feed latency ratio) in males and females. There were no statistically detectable differences in home cage feed latency or any measure of approach latency (Fig1-2). Relative to Veh, desipramine (Dmi) in the drinking water decreased feed latency and test cage:home cage feed latency ratio in males and females after 18 days, but there were no statistically detectable differences as of 8 days or at 8 days or 18 days in home cage feed latency (Fig 1-3A-C). Relative to Veh, Dmi decreased approach latency and test cage:home cage approach latency ratio in females by day 18. There were no statistically detectable differences by 8 days, in home cage approach latency in females or any approach latency in males (Fig 1-3D-F).

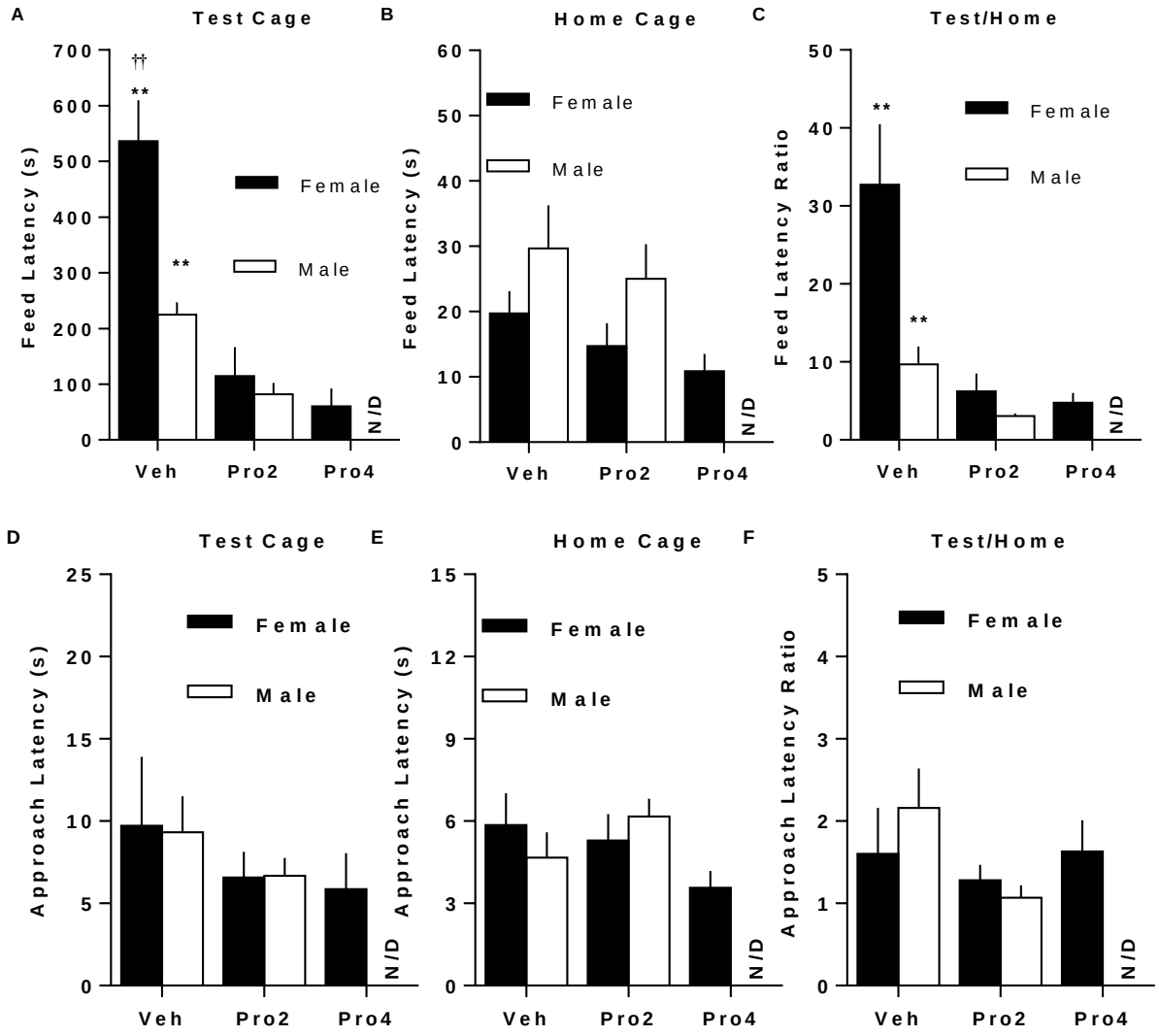

Figure 1-2: **Propranolol (Pro) reduces feed latency in AFEC**. Males (n=6/group) and females (n=7/group) were injected with saline vehicle (Veh), 2 or 4mg/kg Pro and assessed in the AFEC test after 30 minutes. (A) Relative to Veh, Pro reduced feed latency at 2mg/kg in males and both 2 and 4mg/kg in females. Relative to females, males had lower feed latency on Veh and at 2mg/kg Pro. (B) Pro had no statistically detectable effect on home cage feed latency. (C) Relative to Veh, Pro reduced test cage:home cage feed latency ratio in females. (D-F) Pro had no statistically detectable effect on test cage or home cage approach latencies or test cage:home cage approach latency ratio. **p<0.01 between Veh and drug, ††p<0.01 between sexes. (partly from Shannonhouse *et al* 2014b)

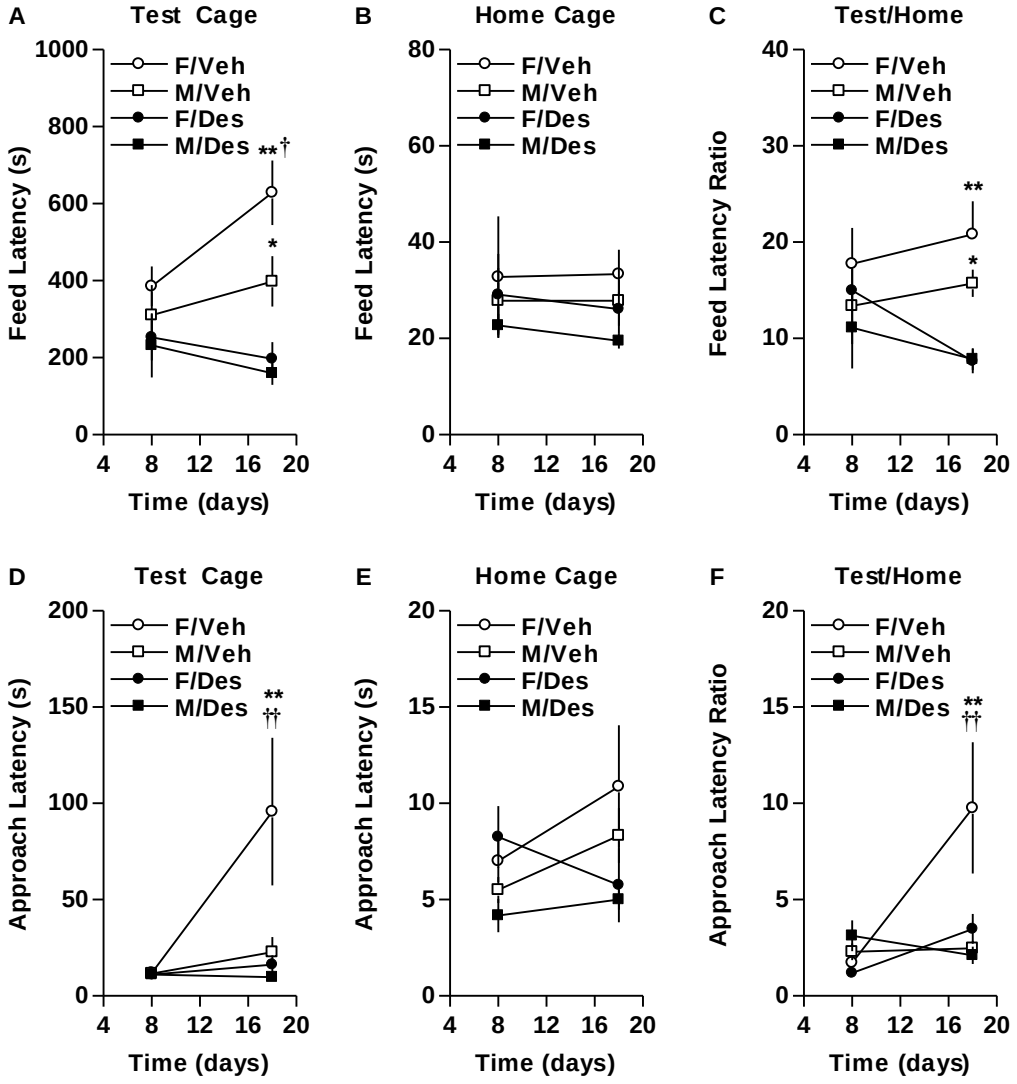

Figure 1-3: **Chronic desipramine reduces test cage feed latency**. Female and male animals (n=8/group) received 0 (vehicle, (Veh)) or 20mg/kg/day desipramine (Des or Dmi) in the drinking water for 18 days. (A) Relative to Veh, Dmi reduced feed latency in both females and males. Feed latency increased in females receiving Veh from day 8 to day 18. (B) Dmi had no statistically detectable effect on home cage feed latency. (C) Relative to Veh, Dmi reduced test cage:home cage feed latency ratio in both females and males on day 18. (D) Relative to day 8, approach latency increased in Veh treated females on day 18. (E) Dmi had no statistically detectable effect on home cage approach latency. (F) Relative to day 8, test cage:home cage approach latency ratio increased in Veh treated females on day 18. *p<0.05, **p<0.01 between Veh and drug, †p<0.05, ††p<0.01 between sexes. (partly from Shannonhouse *et al* 2014b)

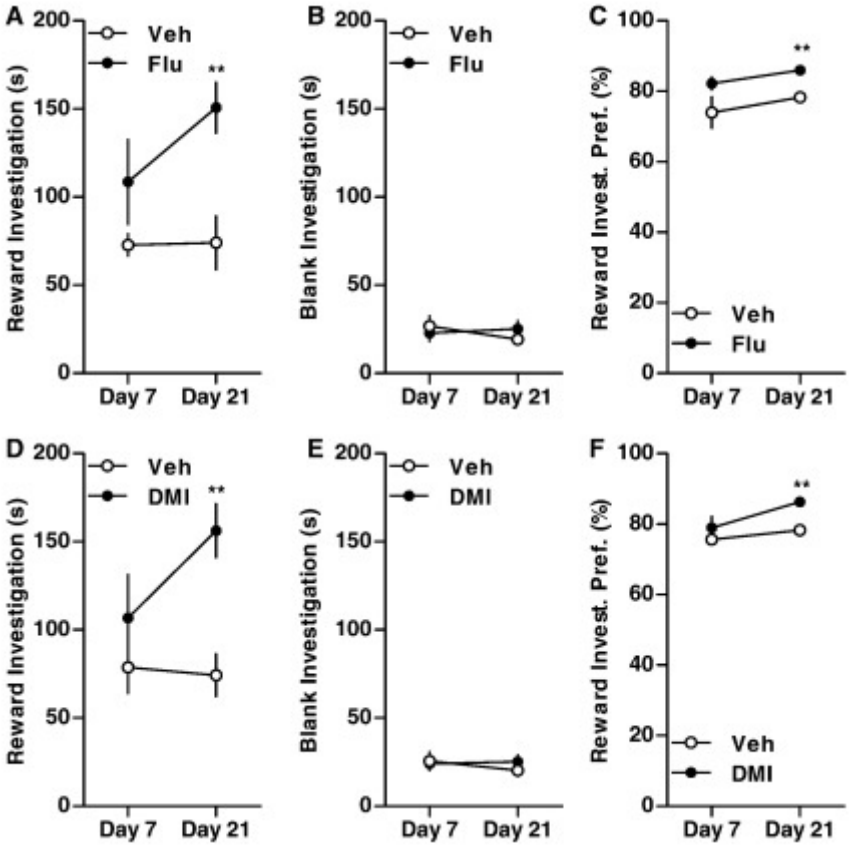

Figure 1-4: **Chronic Fluoxetine (Flu) and Desipramine (Dmi) on RIP**. Female hamsters (n = 6) were subjected to social separation at 5 weeks and administered Flu (0 (Veh) or 10mg/kg/day, po) or Dmi (0 or 20mg/kg/day, po) in the drinking water for 21 days and assessed in RIP on days 7 and 21. Relative to Veh treatment, Flu (A) increased reward investigation time on day 21 but not day 7, (B) did not affect blank investigation time and (C) increased reward preference on day 21 but not day 7. Relative to Veh treatment, Dmi (D) increased reward investigation time on day 21 but not day 7, (E) did not affect blank investigation time and (F) increased reward preference on day 21 but not day 7. Means ± SEM shown. Comparisons by two-way ANOVA drug v vehicle x time. **p<0.01 by post-hoc Bonferroni's t-test. (partly from Shannonhouse *et al* 2016)

Effects of known antidepressants on RIP

The purpose of these experiments was to see if known antidepressants would improve reward investigation and reward preference in RIP. Relative to vehicle (Veh), fluoxetine increased reward investigation and reward preference in females by 21 days, but had no statistically detectable effect on blank investigation at any time point or reward investigation or reward preference at 7 days (Fig 1-4A-C). Relative to Veh, desipramine (Dmi) increased reward investigation and reward preference in females by 21 days, but had no statistically detectable effect on blank investigation at any time point or reward investigation or reward preference at 7 days (Fig 1-4D-F). Relative to Veh, propranolol (Pro), tracazolate (Trac), naltrexone (Nal) and diazepam (Dia) had no statistically detectable effect on reward investigation, blank investigation or reward preference in RIP (Fig 1-5).

Effects of drugs on food intake

The purpose of these experiments was to assess differences in consummatory drive in animals treated with anxiolytics and antidepressants. Relative to vehicle (Veh), 2mg/kg propranolol (Pro) and 10mg/kg Pro had no statistically detectable effect on 2-hour food intake (Fig 1-6A). Relative to Veh, chronic desipramine (Dmi) and fluoxetine (Flu) treatment had no statistically detectable effect on overnight food intake after 1-3 weeks treatment with antidepressant (Fig 1-6B).

Discussion

The purpose of this study was to validate two new behavior tests for Syrian hamsters. For anxiety-related feeding/exploration conflict (AFEC) test, results show treatments known to be anxiolytic (acute diazepam (Dia), acute propranolol (Pro), chronic desipramine (Dmi), chronic fluoxetine (Flu)) reduced test cage but not home cage feed latency. A treatment known to be anxiogenic (acute fluoxetine) worsens performance and a treatment not known to be anxiogenic or anxiolytic (acute Dmi) fails to produce a detectable effect on performance. For

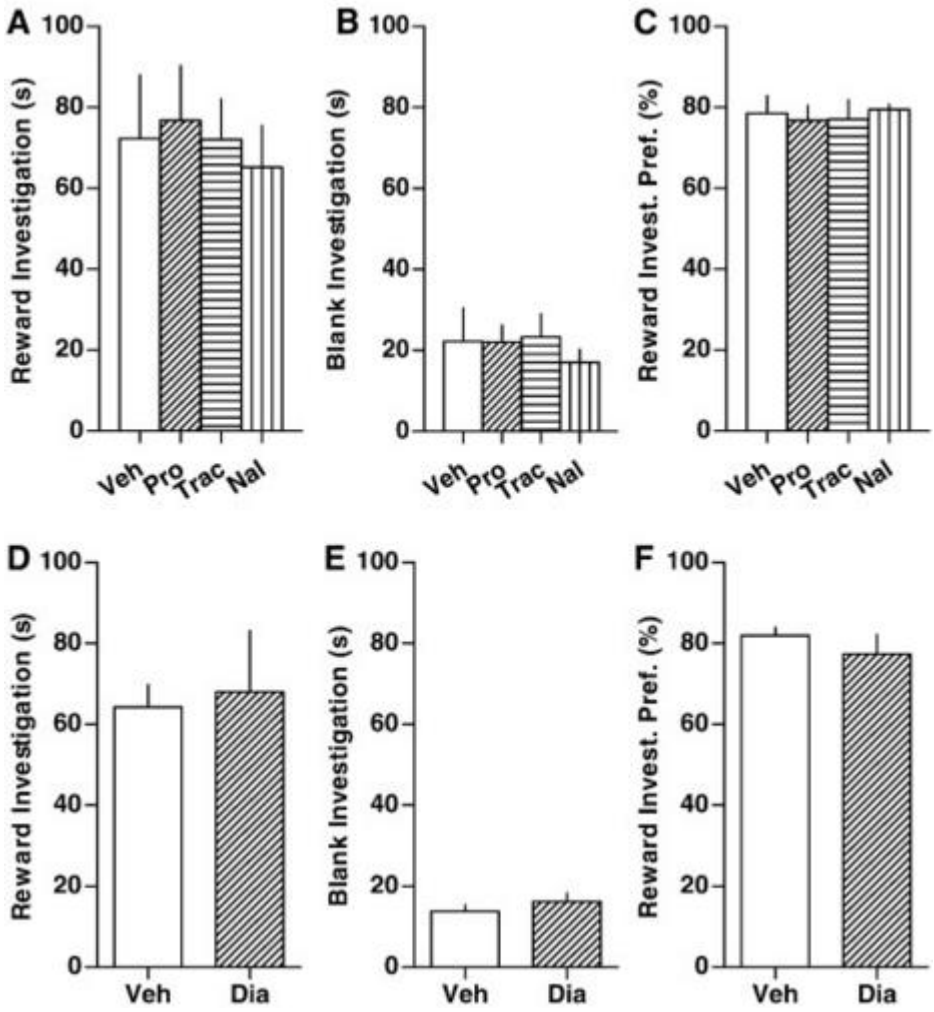

Figure 1-5: **Acute pharmacological control drugs on RIP (propranolol (Pro), tracazolate (Trac), naltrexone (Nal), diazepam (Dia))**. Female hamsters (n = 6) were assessed 30 minutes after intraperitoneal drug injections. (A) Relative to vehicle (Veh), there was no statistically detectable effect on reward investigation by Pro (2mg/kg, ip), Trac (1mg/kg, ip) or Nal (4mg/kg, ip). (B) Relative to Veh, there was no statistically detectable effect on blank investigation by Pro (2mg/kg, ip), Trac (1mg/kg, ip) or Nal (4mg/kg, ip). (C) Relative to Veh, there was no statistically detectable effect on reward preference by Pro (2mg/kg, ip), Trac (1mg/kg, ip) or Nal (4mg/kg, ip). (D-E) There were no statistically detectable effects of Dia (1mg/kg, i.p.) on reward investigation, blank investigation or reward preference. (partly from Shannonhouse *et al* 2014b)

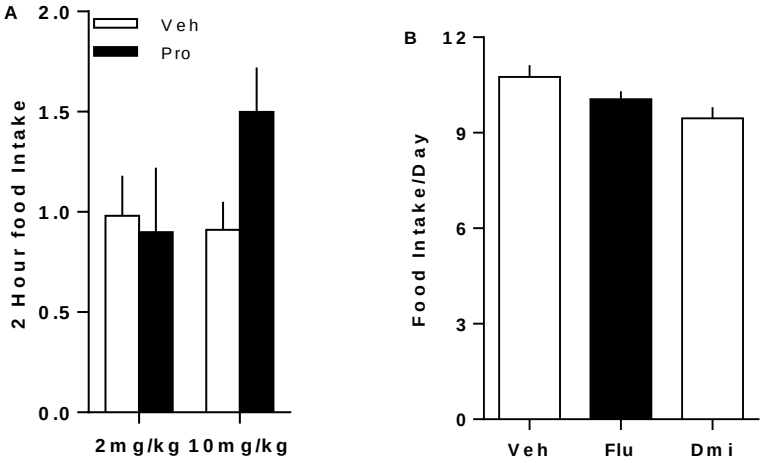

Figure 1-6: **Food intake following propranolol (Pro), desipramine (Dmi) and fluoxetine (Flu)**. (A) Female hamsters socially separated for 5 weeks had feed removed for 2.5 hours and were given intraperitoneal Pro (2mg/kg or 10mg/kg, ip) 30 minutes before food was returned. Two hour food intake is shown. n = 9 for Veh (2mg/kg experiment), n = 9 (2mg/kg Pro), n = 14 veh (10mg/kg Pro), n = 5 (10mg/kg Pro). (B) Socially separated female hamsters were subjected to Veh, Flu (10mg/kg/day, po) or Dmi (20mg/mg/day, po) for 1-3 weeks. Each week, overnight food intake was measured for each animal. n = 9 measurements for Veh, n = 21 measurements for Flu and Dmi. No statistically detectable differences were seen in any experiment. (partly from Shannonhouse *et al* 2014b)

reward investigational preference (RIP) test, chronic antidepressant treatment (Flu or Dmi) improved performance, while acute antidepressant treatment and treatment with 4 drugs not known to affect depression failed to produce a detectable effect on performance.

Cheek pouching is an appetitive, not consummatory, behavior in hamsters (Chester *et al* 2006, Wong and McBride 1993, Arbour and Wilkie 1988). By preventing cheek pouching, we eliminate an appetitive behavior that can decrease feed latency. There was no detectable effect on food intake at the doses of Pro or Dmi used to reduce feed latency and increase reward investigation. Both Pro and Dia were anxiolytic even though they are known to decrease (Wichmann *et al* 2012) and increase (reviewed in Berridge and Peciña 1995), respectively,

appetitive drive and food intake in rodents. The results suggest that AFEC and RIP performance are not affected by changes in consummatory or appetitive drive.

AFEC test performs as predicted in response to antidepressants. Anxiolytic effects of antidepressants require chronic treatment in animal models (Dulawa *et al* 2004, Merali *et al* 2003) and clinical settings (reviewed in Thaler *et al* 2012). Acute Flu treatment can produce anxiogenic-like effects in animal models (Robert *et al* 2011, Silva and Brandão 2000, Silva *et al* 1999), but under some conditions acute treatment can produce anxiolytic-like effects (Rogóż and Skuza 2011, Griebel *et al* 1999). Some patients have initially increased anxiety when treated with Flu (Arrant *et al* 2013, Liu *et al* 2010, Bridge *et al* 2007, Griebal *et al* 1995, Pecknold *et al* 1995, Kshama *et al* 1990). AFEC shows increased anxiety-like effects with acute Flu treatment and decreased anxiety-like effects with chronic Flu or Dmi treatment under these conditions in Syrian hamsters. Furthermore, AFEC can be repeated and drug treated animals still respond as predicted compared to control animals. However, results between trials are not comparable.

Low hedonic drive is a component of depression (Sharpley and Bitsika 2013). Food reward based tests such as progressive ratio (Leventopoulos *et al* 2009, Hodos 1961, reviewed in Stafford *et al* 1998) and sucrose preference (Kubera *et al* 2013, Muscat *et al* 1992, Willner *et al* 1987) are useful is measuring hedonic drive. Disadvantages of progressive ratio include the potentially long duration of a test session, being unreliable in measuring effects of drugs or stressors with short duration effects and subjects becoming sated with or developing tolerance to a reward (reviewed in Stafford *et al* 1998). A disadvantage of sucrose preference is the requirement for a schedule of stress which makes the process both labor-intensive and difficult to perform multiple times on the same animal (Strekalova *et al* 2011).

For Reward/Investigational Preference (RIP) test, results show chronic antidepressant treatment, but not acute treatment, improves performance while treatments not known to have antidepressant effects (Dia, Pro, Trac and Nal)

failed to produce a detectable effect. The test gave consistent results for vehicle treated animals across trials. These results suggest RIP can be repeated without altering results and is a test for chronic antidepressant efficacy. It is unclear if RIP results between trials are comparable.

AFEC and RIP tests are convenient to perform together. They can be performed one after the other in the same cage. AFEC gives the animal time to habituate to the environment prior to RIP. The tests are both low stress (no more stressful than a routine cage change). Anxiety and depression are often comorbid (Grant *et al* 2009) which makes tests for both that can be performed together more appealing.

In conclusion, AFEC and RIP can be used together to measure anxiety-like behavior and hedonic behavior in Syrian hamsters. Tests remain sensitive across repeated use allowing them to be used in repeated measures studies. They involve no more stress than routine cage changes. Blank cassettes, approach latencies and home cage behaviors provide internal controls for exploratory and motoric behaviors. The ability to measure models of comorbid disorders (anxiety and depression) together over time can be a valuable tool for assessing treatments and exacerbating and mitigating factors.

AN EXPERIMENTAL PARADIGM OF SOCIAL HOUSING AND SOCIAL SEPARATION SHOWING FEMALE BIASES IN METABOLISM AND EMOTIONAL STATUS IN SYRIAN HAMSTER (*Mesocricetus auratus*)*

Summary

Anxiety and depression are the two most common types of mood disorders in the United States. Anorexia is comorbid with depression. All three are more prevalent in women than men. The scarcity of animal models linking mood and eating disorders impedes understanding of their biological underpinnings. It is reported here that, relative to socially housed animals (SH, more than one per cage), adult Syrian hamsters housed socially separated (SS, one per cage) induced anorexia characterized by hypophagia, weight loss, decreased adiposity and increased metabolic rate. SS animals increased anxiety-like feed latency in the anxiety feeding/exploration conflict (AFEC) test and thigmotaxis in the open field test. SS animals showed increased depression-like behavior in the reward/investigational preference (RIP) test. SS decreased plasma corticosteroid levels, decreased adrenal mass, decreased hypothalamic corticotropin releasing factor (*Crf*) mRNA and increased hypothalamic mRNA markers of inflammation. All phenotypes showed female bias. Neither adrenal suppression with dexamethasone phosphate (Dx-p) nor Dx-p suppression with corticosteroid replacement could recapitulate the phenotypes in SH males. Relative to SH, SS of weanling animals induced anorexia, anxiety in females and increased mRNA markers of inflammation. Females had decreased hypothalamic *Crf* while *Crf* increased in males. These results show SS hamsters provide a novel experimental paradigm of comorbid anorexia, hypermetabolism, anxiety-like behavior and depression-like behavior.

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*Part of the data reprinted with permission from Female-biased anorexia and anxiety in the Syrian hamster. Shannonhouse *et al*. 2014. Physiology & Behavior.133:141-51.

Introduction

Emotional status plays a major role in energy balance through foraging, consumption, reward, and pyschomotor control of energy expenditure. In male mice stress can increase or decrease weight gain, food intake and caloric efficiency depending on diet (Finger *et al* 2012, Bartolomucci *et al* 2009). Stress decreases effort spent acquiring food reward in male mice (Bartolomucci *et al* 2009) and male and female aged mice (Malatynska *et al* 2012). In male rats stress can decrease weight and food intake and increase anxiety-like and depression-like behaviors (Iio *et al* 2012, Rygula *et al* 2005). In male hamsters stress social stress increased food intake, body weight and adiposity (Foster *et al* 2006) while foot shock stress increases body mass and adiposity (Solomon *et al* 2007). In obese humans depression and hysteria are associated with increased sweet taste preference relative to salty (Aguayo *et al* 2012). Compared to men with major depressive disorder, women with major depressive disorder have elevated rates of obesity and bulimia (Marcus *et al* 2008). Despite higher rates of anxiety and depression in women than men, most behavior experiments in animal models use males, which limits the ability to elucidate the biological underpinnings of sex differences (reviewed in Donner and Lowry 2013). More animal models of emotional status and energy balance integrating females and sex differences would help address this knowledge gap.

This section presents an experimental paradigm in Syrian hamsters (*Mesocricetus auratus*) linking moving animals from social housing to social separation with female-biased voluntary food restriction, increased metabolic rate, decreased hypothalamic-pituitary-adrenal axis (HPA) activity, decreased adiposity and lean mass and increased anxiety-like and depression-like behavior. Experiments address possible mechanisms using inflammation-related gene expression and HPA activity and manipulating age of separation and HPA activity. The discussion compares merits of the model to related models in other animals.

Anxiety and depression are more likely to affect women than men (Grant *et al* 2015, Grant *et al* 2009, Bjil *et al* 2002). Both anxiety and depressive disorders are comorbid with eating disorders (Grant *et al* 2009, Hudson *et al* 2007). Eating disorders predict increased risk for later depression and depression predicts increased risk for later eating disorders (Puccio *et al* 2016). Contemporaneously with this dissertation project, researchers used the adolescent activity based anorexia (ABA) paradigm with restricted feeding schedule and wheel running activity to model anorexia nervosa in adulthood. In female rats, ABA increases anxiety and increases HPA activity (Kinzig and Hargrave 2010). The ABA paradigm showed an association between changes in γ–amino butyric acid (GABA) signaling in anorexia resistant mice relative to mice that developed anorexia (Chen *et al* 2015, Aoki *et al* 2012, reviewed in Aoki 2016). No paper mentions ABA in males. The success of ABA in experimentally addressing neurological mechanisms linking metabolism, stress and behavior highlights the clinical relevance of using female animal models in studying links between emotional status and metabolism.

The degree of control over stressors plays an important role in effects stressors have on behavior. In a comparison of unstressed, inescapably stressed and escapably stressed human subjects found escapable stress lessens fear and improves fear extinction while inescapable stress heightens fear and reduces fear extinction (Hartley *et al* 2014). Stress improves behavioral outcomes when it is escapable in rats (Rozeske *et al* 2012), mice (Sanford *et al* 2010) and Syrian hamsters (Cordner *et al* 2004). Controlling food intake by limiting available food (e.g., ABA) represents an uncontrollable stressor. Therefore, a model of volitional control of food intake would be helpful in distinguishing emotional status effects of no control over food intake from food intake *per se*.

Loss of social contact or perceived loss of social contact is associated with anxiety and depression in humans (reviewed in Shear and Skritsaya 2012, Kawachi and Berkman 2001). Social separation of male rats is anxiogenic (Das *et*

al 2016, Wallace *et al* 2009, Sherif *et al* 1995, Ahmed *et al* 1995) and increases depression-like behavior (Das *et al* 2016). In prairie voles social separation increases anxiety like (Grippo *et al* 2008) and depression like behaviors in females (Grippo *et al* 2014) and decreases anxiety in males (Stowe *et al* 2005). Further searching is warranted for an animal model of female biased separation-induced anxiety-like and depression-like behaviors.

Materials and Methods

Animals

Syrian hamsters (*Mesocricetus auratus*) of the Lake View Gorge strain (Charles River, Kingston, NY) were purchased for use at age 8 weeks or were bred in the Kleberg Laboratory Animal Facility at Texas A&M University. Animals were kept on a 14h:10h light:dark schedule (lights on at 0600h) at 23 ± 3˚C. LabDiet 5001 (Purina, Richmond, IN) and water were provided ad libitum. Food intake was monitored by weighing food from the food hopper, the bedding and cheek pouches to the nearest 0.1g. Bedding was Sani-Chips (Murphy's Products, Monteville, NJ). Each cage was supplied with Nestlets (Ancare, Bellmore, NY). Females and males were group housed until postnatal day 70 (PD70) when they were subjected to social housing (SH, 2 or more per cage) or social separation (SS, 1 per cage). Juvenile experiments (SH or SS from PD29) were conducted similarly. Tissues were collected by killing the animals by beheading. Fat pads, anterior tibialis and tibias were weighed and flash frozen in liquid nitrogen. Brains were frozen in isopentane pre-chilled with dry ice. Procedures used were approved by the Institutional Animal Care and Use Committee.

Anxiety-related feeding/exploration conflict test (AFEC test)

AFEC was performed as described previously (Section 2, Shannonhouse *et al* 2014b). Briefly, an animal is placed in a polycarbonate cage on a white surface in ambient light of ~1000 lux. A plexoglass cover with a hole in the center is placed over the cage to prevent climbing out and graham cracker in a spring loaded clamp is suspended through the hole. Approach and feed latencies were timed.

Reward/investigational preference test (RIP test)

RIP was performed as described previously (Section 2, Shannonhouse *et al* 2015). Briefly, clear plastic cases containing either graham cracker (reward) or nothing (blank) were either placed in the food hopper of a polycarbonate test cage or suspended through a hole in a clear plastic cover in a spring loaded clamp. Time spent investigating (sniffing, chewing, scratching) each case was measured. Reward investigation time and reward investigational preference (100% * reward investigation time / [reward + blank investigation time]) were used as indices of hedonic drive.

Indirect calorimetry

Animals were assessed in an Oxymax System (Columbus Instruments, Columbus, OH). Sampled air has passed through CO_2 and O_2 sensors to measure the decrease and increase in O_2 and CO_2 leaving the test chamber relative to O_2 and CO_2 entering the test chamber. The difference was used to calculate heat produced and respiratory exchange ratio. Metabolic rate was estimated by [heat production / body mass$^{2/3}$].

Microdissection

Frozen brains were blocked, mounted on a freezing microtome stage and sectioned at 150µm. Tissue sections were placed on glass slides on a freezing plate. Using the Hamster Brain Atlas (Morin and Wood 2001) as a guide, specific brain regions were dissected under a dissecting microscope using 0.75mm metal core punches (Ted Pella, Redding CA).

RNA extraction

RNA was extracted using a modified guanidinium isothiocyanate method as described previously (Morgan *et al* 2003). Briefly, tissues were homogenized in a buffered 4M guanidinium isothiocyanate, sodium lauroyl sarcosine solution and RNA was precipitated in 3M LiCl and pelleted by centrifugation. Protein in the pellets was digested with proteinase K, followed by phenol/chloroform extraction,

and ethanol precipitation. RNA was extracted with acidic phenol to reduce DNA contamination before reverse transcription.

Reverse transcription PCR

Reverse transcription was performed using Moloney murine leukemia virus reverse transcriptase (M-MLV) with random primers (New England Biolabs, Ipswich, MA) according to manufacturer's instructions. PCR reactions were performed using 2X PCR mix (New England Biolabs, Ipswich, MA). A pre-amplification PCR was performed containing primers for low-abundance *Crf*, *Il6* and *Tlr4* cDNA (Table 2-5) followed by a main PCR for 26 cycles containing primers for the gene of interest plus *Actb* as a standard.

Radioimmunoassays

Serum corticosterone and cortisol were measured using Coat-aCount RIA kits (Diagnostic Products Corporation, Los Angeles, CA). Intra-assay variation: 10% and 5%, respectively. Limits of detection were <0.8ng/mL and 0.5ng/mL, respectively. Samples were assayed in duplicate.

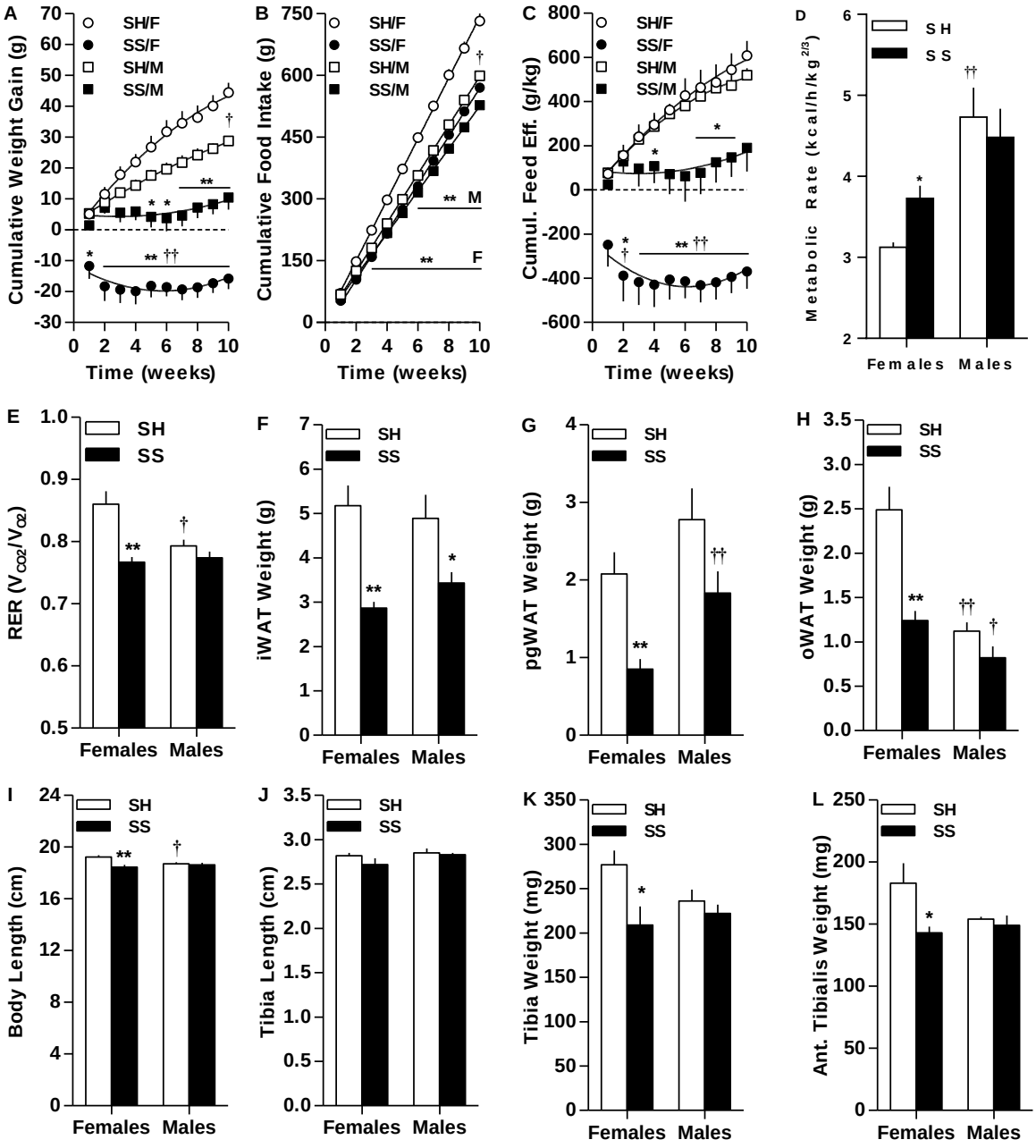
Drugs

Drugs were purchased from Sigma-Aldrich (St Louis, MO). Drugs were dissolved in the drinking water as described: dexamethasone phosphate (Dx-p, 4.7µg/mL), Dx-p plus cortisol (D+F, 4.7 and 1.2µg/mL) or Dx-p plus corticosterone (D+B, 4.7 and 1.2µg/mL) for 250µg/kg/day, p.o., 250 and 50µg/kg/day, p.o. and 250 and 50µg/kg/day, p.o., respectively assuming water intake of 8mL/day (Lee *et al* 2001, Richardson *et al* 1992). Drinking water was replaced every 3 days.

Statistical analysis

Two-way ANOVA (Fig 2-1D-L; Fig 2-4D-I; Fig 2-5) followed by Bonferroni's t-test, Student's t-test (Fig 2-3) and one-way ANOVA (Fig 2-6 G-I) followed by Bonferroni's t-test were performed using Prism 5.04 and InStat 3.00 (GraphPad, San Diego, CA). Repeat measures 2-way ANOVA (Fig 2-1A-C; Fig 2-2; Fig 2-4A-C; Fig 2-6 A-F) was performed using SAS Enterprise Guide 6.4 (SAS, Cary, North Carolina).

Figure 2-1: **Effects of social housing v social separation on growth, food intake, metabolic rate and tissue masses**. Female and male hamsters were either socially housed (SH) or socially separated (SS) (n = 8/group, except SS Males n = 9). Food intake sample sizes are n = 4 for both SH groups. Body weight and food intake were monitored weekly. Metabolism was assessed by indirect calorimetry after 8 weeks. Tissues were collected at the end of the experiment. (A) Relative to SH, SS reduced body weight gain. The effect was more pronounced in females. (B) Relative to SH, SS reduced food intake. The effect was more pronounced in females. (C) Relative to SH, SS reduced feed efficiency. The effect was more pronounced in females. (D) Relative to SH, SS increased metabolic rate in females. SH males had a higher metabolic rate than SH females. (E) Relative to SH, SS decreased RER in females. SH males had a lower RER than SH females. (F) Relative to SH, SS decreased inguinal white adipose tissue mass. (G) Relative to SH, SS decreased perigonadal white adipose tissue mass in females. (H) Relative to SH, SS decreased omental white adipose tissue mass in females. Relative to females, omental white adipose tissue mass was lower in males. (I) Relative to SH, SS decreased body length in females. Relative to SH females, SH males had shorter body lengths. (J) There was no statistically detectable effect of housing or sex on tibia length. (K-L) Relative to SH, SS decreased tibia and anterior tibialis weights in females. *$p<0.05$, **$p<0.01$ between housing conditions, †$p<0.05$, ††$p<0.01$ between sexes. (from Shannonhouse *et al* 2014a)


A
Cumulative Weight Gain (g)
SH/F
SS/F
SH/M
SS/M
Time (weeks)
B
Cumulative Food Intake (g)
M
F
Time (weeks)
C
Cumul. Feed Eff. (g/kg)
Time (weeks)
D
Metabolic Rate (kcal/h/kg2/3)
SH
SS
Females Males
E
RER (VCO2/VO2)
SH
SS
Females Males
F
iWAT Weight (g)
Females Males
G
pgWAT Weight (g)
Females Males
H
oWAT Weight (g)
Females Males
I
Body Length (cm)
Females Males
J
Tibia Length (cm)
Females Males
K
Tibia Weight (mg)
Females Males
L
Ant. Tibialis Weight (mg)
Females Males

Results

Effects and interactions of social housing conditions and sex on energy balance in adult animals

Female and male hamsters were subjected to social housing (SH) or social separation (SS) for 10 weeks while measuring body weight and food intake. Relative to SH, from weeks 1-10 SS females showed decreased cumulative body weight gain, decreased cumulative food intake and decreased feed efficiency (Table 2-1, Fig 2-1 A-C). Relative to SH, SS males showed decreased cumulative body weight gain from weeks 4-10, decreased cumulative food intake from weeks 7-10 and decreased feed efficiency from weeks 5-10. Relative to females, males ate less in a social housing conditions dependent manner (Table 2-1, Fig 2-1 B). Relative to SH females, SH males showed decreased weight gain and relative to SS females, SS males showed increased weight gain (Table 2-1, Fig 2-1A).
A second cohort of animals was subjected to SH or SS for 8 weeks and assessed by indirect calorimetry to determine metabolic effects of social housing conditions. Relatives to females, males had increased metabolic rate per surface area (estimated by body mass$^{2/3}$) and relative to SH females, SS females had increased basal metabolic rate/surface area (Fig 2-1D). Respiratory exchange ratio (RER) was used to estimate the relative utilization of carbohydrates v fats (Jasson 1982). Relative to SH, SS females showed decreased RER (indicating more energy coming from fats and less from carbohydrates) and relative to SH females, SH males showed decreased RER (Fig 2-1E).

After 20 weeks of SH or SS, animals from the second cohort were sacrificed to measure indicators of body composition. Relative to SH females, SS females had decreased fat pad masses of inguinal, perigonadal and omental white adipose fat pads. Relative to SH males, SS males had decreased inguinal fat pad mass. Compared to SH females, SH males had decreased omental fat pad mass. Compared to SS females, SS males had increased perigonadal and decreased omental fat pad masses (Fig 2-1F-H). Compared to SH females, SS females had

Table 2-1: Body weight gain, food intake and feed efficiency RM ANOVA on adult females and males subjected to SH or SS from postnatal day 29		
	F Statistic	p-value
Cumulative Weight Gain		
Housing	$F_{1,112} = 24.43$	<0.01
Sex	$F_{1,112} = 0.74$	0.392
Time	$F_{1,112} = 64.34$	<0.01
Housing x Sex	$F_{1,112} = 3.29$	0.072
Housing x Time	$F_{1,112} = 0.24$	0.870
Sex x Time	$F_{1,112} = 0.24$	0.441
Housing x Sex x Time	$F_{1,112} = 0.53$	0.663
Cumulative Food Intake		
Housing	$F_{1,112} = 33.00$	<0.01
Sex	$F_{1,112} = 0.53$	0.469
Time	$F_{1,112} = 749.32$	<0.01
Housing x Sex	$F_{1,112} = 2.54$	0.114
Housing x Time	$F_{1,112} = 4.38$	<0.01
Sex x Time	$F_{1,112} = 0.11$	0.951
Housing x Sex x Time	$F_{1,112} = 0.12$	0.946
Cumulative Feed Efficiency		
Housing	$F_{1,112} = 10.47$	<0.01
Sex	$F_{1,112} = 2.68$	0.104
Time	$F_{1,112} = 71.79$	<0.01
Housing x Sex	$F_{1,112} = 0.87$	0.352
Housing x Time	$F_{1,112} = 0.20$	0.894
Sex x Time	$F_{1,112} = 0.33$	0.801
Housing x Sex x Time	$F_{1,112} = 0.50$	0.683

Table 2-1: **Body weight gain, food intake and feed efficiency RM ANOVA on adult females and males subjected to SH or SS from postnatal day 29.** Animals assessed weekly for body weight and food intake for 4 weeks. (from Shannonhouse *et al* 2014a)

Figure 2-2: **Effects of social housing on anxiety**. (A-C) Female and male SH and SS hamsters (n = 6/group) were subjected to the anxiety feeding/exploration conflict test. (A) Relative to SH animals, SS animals show increased feed latency by 4 or 6 weeks (for females and males, respectively). (B) There was no statistically detectable effect of housing or sex on home cage feed latency. (C) Relative to SH animals, SS animals show increased test cage:home cage feed latency ratio by 4 or 6 weeks (for females and males, respectively). (D-I) Female and male SH and SS hamsters (n = 6/group except SS females where n = 4) were subjected to the open field test. (D) Relative to SH animals, SS animals spent less time in the center of the cage. (E) Relative to SH animals, SS animals spent more time in the periphery of the cage. (F) Relative to SH animals, SS males spent a lower percentage of time in the center of the cage. (G-I) There were no statistically detectable effects on center activity, periphery activity or percentage center activity by sex or housing conditions. *p<0.05, **p<0.01 between housing conditions, †p<0.05, ††p<0.01 between sexes. (from Shannonhouse *et al* 2014a)

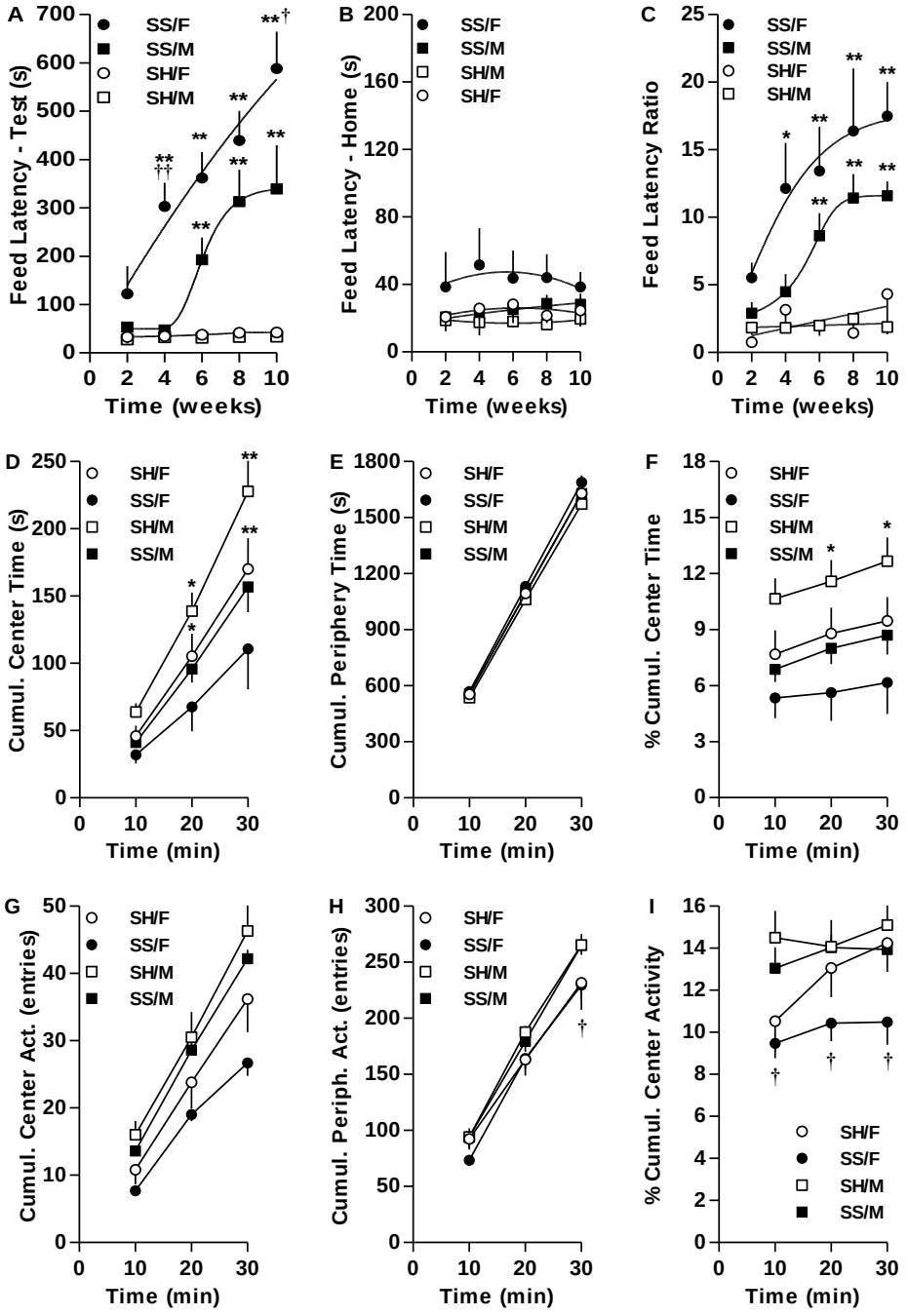

A
Feed Latency - Test (s)
Time (weeks)
SS/F
SS/M
SH/F
SH/M
B
Feed Latency - Home (s)
Time (weeks)
C
Feed Latency Ratio
Time (weeks)
D
Cumul. Center Time (s)
Time (min)
E
Cumul. Periphery Time (s)
Time (min)
F
% Cumul. Center Time
Time (min)
G
Cumul. Center Act. (entries)
Time (min)
H
Cumul. Periph. Act. (entries)
Time (min)
I
% Cumul. Center Activity
Time (min)

Table 2-2: AFEC test cage feed latency, home cage feed latency and feed latency ratio RM ANOVA on SH and SS adult females and males		
	F Statistic	p-value
Test Cage Feed Latency		
Housing	$F_{1,100} = 23.87$	<0.01
Sex	$F_{1,100} = 173.83$	<0.01
Time	$F_{1,100} = 14.03$	<0.01
Housing x Sex	$F_{1,100} = 20.65$	<0.01
Housing x Time	$F_{1,100} = 12.94$	<0.01
Sex x Time	$F_{1,100} = 0.87$	0.483
Housing x Sex x Time	$F_{1,100} = 0.89$	0.476
Home Cage Feed Latency		
Housing	$F_{1,100} = 7.45$	<0.01
Sex	$F_{1,100} = 6.88$	0.01
Time	$F_{1,100} = 0.11$	0.980
Housing x Sex	$F_{1,100} = 1.67$	0.199
Housing x Time	$F_{1,100} = 0.09$	0.986
Sex x Time	$F_{1,100} = 0.20$	0.938
Housing x Sex x Time	$F_{1,100} = 0.11$	0.982
Test Cage:Home Cage Feed Latency Ratio		
Housing	$F_{1,100} = 103.12$	<0.01
Sex	$F_{1,100} = 12.20$	<0.01
Time	$F_{1,100} = 6.37$	<0.01
Housing x Sex	$F_{1,100} = 8.67$	<0.01
Housing x Time	$F_{1,100} = 4.88$	<0.01
Sex x Time	$F_{1,100} = 0.48$	0.751
Housing x Sex x Time	$F_{1,100} = 0.15$	0.961

Table 2-2: **AFEC test cage feed latency, home cage feed latency and feed latency ratio RM ANOVA on SH and SS adult females and males**. Animals were subjected to SH or SS and tested every 2 weeks. (from Shannonhouse *et al* 2014a)

Table 2-3: **Open field RM ANOVA on SH and SS adult females and males**. Animals were subjected to SH or SS and for 8 weeks. Scores were assessed in 10 minute bins. (from Shannonhouse *et al* 2014a)

Table 2-3: Open field RM ANOVA on SH and SS adult females and males		
	F Statistic	p-value
Cumulative Center Time		
Housing	$F_{1,54} = 19.07$	<0.01
Sex	$F_{1,54} = 11.47$	<0.01
Time	$F_{1,54} = 54.84$	<0.01
Housing x Sex	$F_{1,54} = 0.20$	0.654
Housing x Time	$F_{1,54} = 2.05$	0.138
Sex x Time	$F_{1,54} = 1.37$	0.264
Housing x Sex x Time	$F_{1,54} = 0.01$	0.990
Cumulative Periphery Time		
Housing	$F_{1,54} = 19.01$	<0.01
Sex	$F_{1,54} = 11.34$	<0.01
Time	$F_{1,54} = 4291.10$	<0.01
Housing x Sex	$F_{1,54} = 0.19$	0.665
Housing x Time	$F_{1,54} = 2.01$	0.144
Sex x Time	$F_{1,54} = 1.34$	0.272
Housing x Sex x Time	$F_{1,54} = 0.01$	0.989
Cumulative % Time in Center		
Housing	$F_{1,54} = 23.12$	<0.01
Sex	$F_{1,54} = 13.56$	<0.01
Time	$F_{1,54} = 1.80$	0.176
Housing x Sex	$F_{1,54} = 0.36$	0.549
Housing x Time	$F_{1,54} = 0.06$	0.946
Sex x Time	$F_{1,54} = 0.07$	0.935
Housing x Sex x Time	$F_{1,54} = 0.05$	0.953
Cumulative Activity in Center		
Housing	$F_{1,54} = 3.68$	0.060
Sex	$F_{1,54} = 6.46$	0.014
Time	$F_{1,54} = 44.66$	<0.01
Housing x Sex	$F_{1,54} = 0.56$	0.458
Housing x Time	$F_{1,54} = 0.29$	0.749
Sex x Time	$F_{1,54} = 0.37$	0.696
Housing x Sex x Time	$F_{1,54} < 0.01$	0.996
Cumulative Activity in Periphery		
Housing	$F_{1,54} = 0.10$	0.750
Sex	$F_{1,54} = 0.01$	0.924
Time	$F_{1,54} = 72.47$	<0.01
Housing x Sex	$F_{1,54} = 3.02$	0.088
Housing x Time	$F_{1,54} = 0.08$	0.928
Sex x Time	$F_{1,54} = 0.01$	0.994

Table 2-3 continued		
Housing x Sex x Time	$F_{1,54} = 0.78$	0.466
Cumulative % Activity in Center		
Housing	$F_{1,54} = 4.88$	0.031
Sex	$F_{1,54} = 12.98$	<0.01
Time	$F_{1,54} = 1.99$	0.146
Housing x Sex	$F_{1,54} = 0.72$	0.400
Housing x Time	$F_{1,54} = 0.18$	0.834
Sex x Time	$F_{1,54} = 0.15$	0.863
Housing x Sex x Time	$F_{1,54} = 1.12$	0.334

shorter bodies, decreased tibia masses and decreased anterior tibialis masses. Compared to SH females, SH males had shorter bodies (Fig 2-1I-J).

Effects and interactions of social housing conditions and sex on anxiety, locomotor activity and hedonic drive in adult animals

Animals were subjected to SH or SS from 10 weeks of age and anxiety was assesed using anxiety feeding/exploration conflict test (AFEC, Shannonhouse *et al* 2014b) every two weeks and open field test after 8 weeks to asses anxious and locomotor behavior (Table 2-3). Relative to SH, SS increased test cage feed latency and test cage:home cage feed latency ratio after 4 weeks in females and 6 weeks in males in AFEC (Fig 2-2A-C, Table 2-2). Relative to SH, SS females and males spent less time in the center and more time in the periphery (Fig 2-2D-E). However, as a proportion the difference was only statistically distinguishable in males (Fig 2-2F). There were no statistically detectable differences in entries into the center or periphery (Fig2-2G-I).

After 8 weeks of SH or SS, female and male animals were subjected to reward/investigational preference (RIP) test to assess hedonic drive. Compared to SH females, SS females decreased reward cassette investigation time. Males spent more time investigating the reward cassette than females, but there were no statistically detectable differences between SH males and SS males (Fig 2-3A). There were no statistically detectable differences in blank investigation (Fig 2-3B). Compared to SH females, SS females showed decreased reward preference. Compared to SS females, SS males showed increased reward preference (Fig 2-3C).

Effect of social housing conditions and sex on energy balance, anxiety and hedonic drive in juvenile animals

Female and male animals were subjected to SH or SS from postnatal day 29 (PD29) and assessed for body weight gain and food intake (Table 2-4). Compared to SH females, SS females gained less weight, ate less and had lower feed efficiency. Compared to SH males, SS males had low food intake, but there

were no statistically detectable differences in body weight gain or feed efficiency (Fig 2-4A-C).

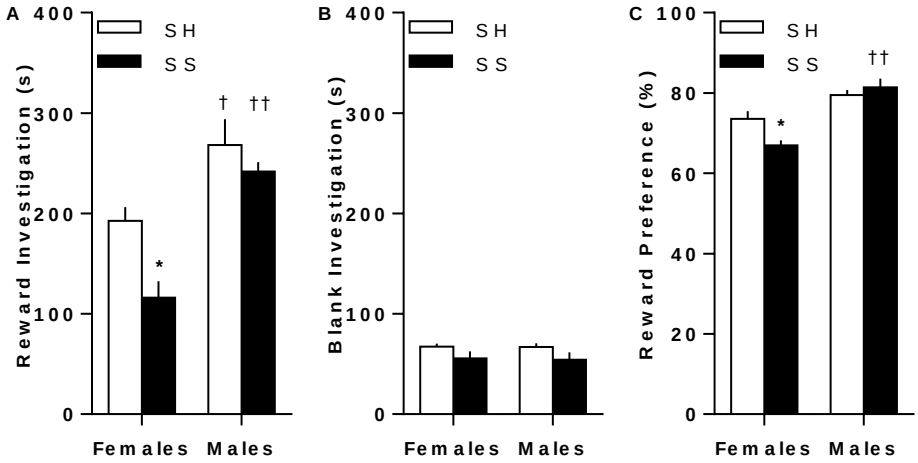


Figure 2-3: **Effects of social housing on hedonic drive**. Female and male SH and SS animals (n = 8/group) were subjected to the reward/investigational preference test after 10 weeks. (A) Relative to SH, SS females showed less reward investigation. Relative to females, males showed higher reward investigation. (B) There was no statistically detectable effect on blank investigation. (C) Relative to SH, SS females showed lower reward preference. Relative to SS females, SS males showed higher reward preference. *p<0.05 between housing conditions, †p<0.05, ††p<0.01 between sexes. (partly from Shannonhouse *et al* 2014a)

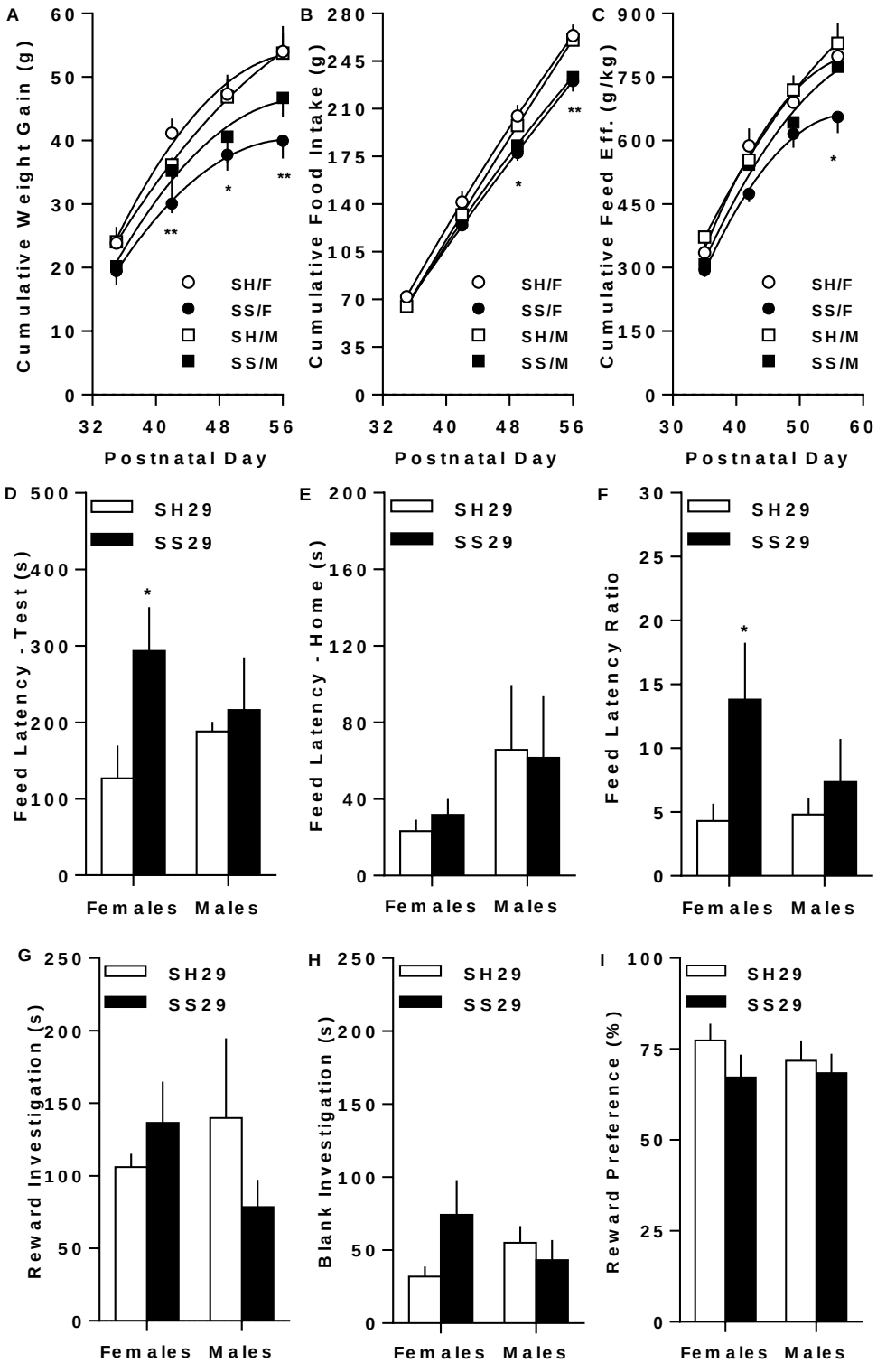
Table 2-4: AFEC and RIP in socially housed (SH) v socially separated (SS) females		
	F Statistic	p-value
AFEC, Feed Latency		
Housing (SH v SS)	$F_{1,20} = 5.49$	$p = 0.03$
Age (SH v SS at PD70 v PD29)	$F_{1,20} = 152.5$	$p < 0.01$
Housing x Age	$F_{1,20} = 8.29$	$p < 0.01$
AFEC, Feed Latency Ratio		
Housing (SH v SS)	$F_{1,20} = 64.87$	$p < 0.01$
Age (SH v SS at PD70 v PD29)	$F_{1,20} = 1.49$	$p > 0.05$
Housing x Age	$F_{1,20} = 0.05$	$p > 0.05$
RIP, Reward Investigation Time		
Housing (SH v SS)	$F_{1,20} = 12.17$	$p < 0.01$
Age (SH v SS at PD70 v PD29)	$F_{1,20} = 25.49$	$p < 0.01$
Housing x Age	$F_{1,20} = 65.85$	$p < 0.01$
RIP, Reward Preference		
Housing (SH v SS)	$F_{1,20} = 35.68$	$p < 0.01$
Age (SH v SS at PD70 v PD29)	$F_{1,20} = 2.19$	$p > 0.05$
Housing x Age	$F_{1,20} = 1.69$	$p > 0.05$

Table 2-4: AFEC and RIP in socially housed (SH) v socially separated (SS) females. Animals were socially housed until ages PD70 or PD29 and subjected to SH or SS for 4 weeks. (from Shannonhouse *et al* 2014a)

Females and males were subjected to SH and SS from PD29 for 4 weeks and tested in AFEC and RIP (Table 2-4). Compared to SH females, SS females had lower feed latency and test cage:home cage feed latency ratio. There were no statistically detectable differences between SH and SS males (Fig 2-4D-F). RIP tests showed no statistically detectable differences by housing or sex (Fig 2-4G-I).

Females subjected to SH and SS from PD29 behaved differently than females subjected to SH and SS from PD70 (Table 2-4). Age x Housing Interaction explained more variation than age in AFEC feed latency, but housing conditions explained far more variation than age and age x housing combined (Table 2-4). In contrast, only housing conditions produced a statistically detectable effect in AFEC feed latency ratio. Age x Housing Interaction explained more

Figure 2-4: **Effect on social housing on adolescent female and male hamsters**. Female and male hamsters SH or SS at 29 days old (PD29) (n = 4-7/group except for SH food intake where n = 3) were subjected to AFEC and RIP. (A) Relative to SH females, females SS from PD29 gained less weight. Weight gain in males SH and SS from PD29 were not statistically distinguishable. (B) Relative to SH animals, SS from PD29 males and females ate less food. (C) Relative to SH, females SS from PD29 had lower feed efficiency. (D) Relative to SH females, SS from PD29 females showed higher feed latency. SH and SS from PD29 male feed latencies were not statistically distinguishable. (E) Home cage feed latencies were not statistically distinguishable. (F) Relative to SH females, SS from PD29 females showed higher test cage:home cage feed latency ratio. SH and SS from PD29 male feed latency ratios were not statistically distinguishable. (G-I) There were no statistically detectable effects of sex or SS from PD29 in the RIP test. *$p<0.05$, **$p<0.01$ between housing conditions, †$p<0.05$ between sexes. (partly from Shannonhouse *et al* 2014a)


A
Cumulative Weight Gain (g)
Postnatal Day
SH/F
SS/F
SH/M
SS/M
B
Cumulative Food Intake (g)
C
Cumulative Feed Eff. (g/kg)
D
Feed Latency - Test (s)
SH29
SS29
Females Males
E
Feed Latency - Home (s)
F
Feed Latency Ratio
G
Reward Investigation (s)
H
Blank Investigation (s)
I
Reward Preference (%)

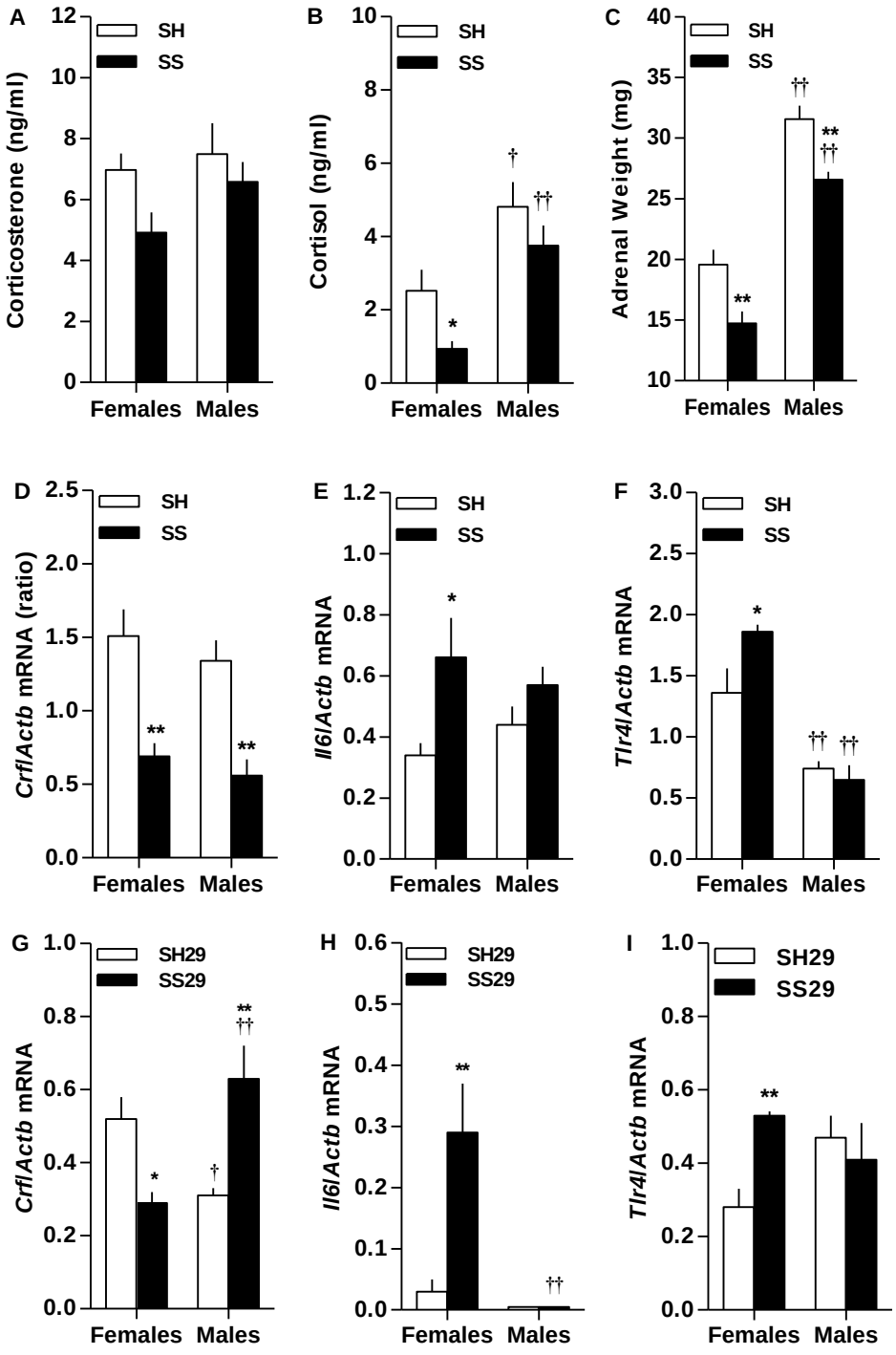
variation in RIP reward investigation time than age and housing combined (Table 2-4). In contrast, only housing conditions produced a statistically detectable effect in RIP-reward preference.

Effect of social housing conditions, sex and age on HPA function and RNA immune markers

This experiment assessed differences in hypothalamic-pituitary-adrenal axis (HPA) activity and brain immune markers predicted to be affected by HPA activity with respect to SH v SS in females and males. Tissues from animals subjected to SH or SS from PD70 for 10 weeks. Compared to SH females, SS females had reduced cortisol and lower adrenal weights. Compared to SH males, SS males had reduced adrenal weight. Compared to females, males had higher cortisol and higher adrenal weights. There were no statistically detectable differences in corticosterone (Fig 2-5A-C).

RNA extracted from the hypothalamai of animals subjected to SH or SS from PD70 for 10 weeks or PD29 for 4 weeks was assessed for HPA and immune markers by RT-PCR. Compared to SH, SS from PD70 females and males had decreased corticotropin releasing factor (*Crf*) mRNA (Fig 2-5D). Compared to SH females, SS from PD70 females had increased interleukin 6 (*Il6*) mRNA (Fig 2-5E). Compared to SH females, SS from PD70 females had increased toll-like receptor 4 (*Tlr4*) mRNA. Compared to females, males had decreased *Tlr4* mRNA (Fig 2-5F). The gene expression profiles from animals SH or SS from PD29 were qualitatively different. Compared to SH, SS from PD29 females had decreased *Crf* mRNA but males had increased *Crf* mRNA (Fig 2-5G). *Il6* mRNA was much higher in females SS from PD29 than SH females or any males to the point *Il6* was often undetectable in the other groups (Fig 2-5H). Compared to SH females, SS females had increased *Tlr4* mRNA (Fig 2-5I).

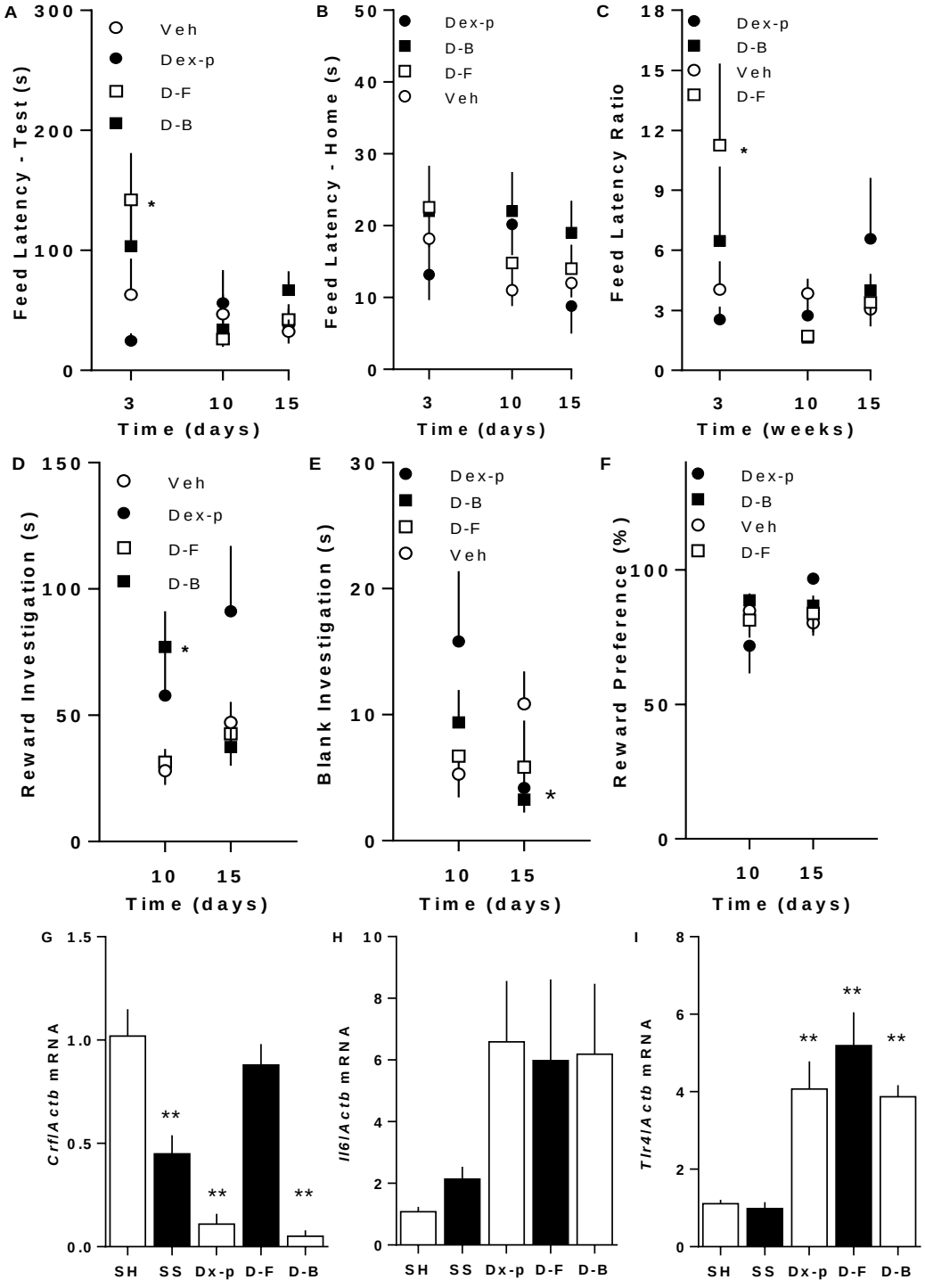
Figure 2-5: **Adrenal steroids and hypothalamic gene expression in socially housed and socially separated hamsters**. Female and male hamsters were subjected to SH or SS for 8 weeks from PD70 or PD29. (A-C) Adrenal steroids and weights in animals SH or SS from PD70. (A) Plasma corticosterone was unaffected by housing or sex (PD70 animals only). (B) Relative to SH, SS lower plasma cortisol in females. There was no statistically detectable effect of SS on cortisol levels in males. Males had higher cortisol levels than females. (C) Relative to SH, SS reduced adrenal weight. Males had larger adrenals than females. (D-F) Hypothalamic gene expression in animals SH or SS from PD70. (D) Relative to SH, SS from PD70 reduced *Crf*/*Actb* mRNA. (E) Relative to SH, SS from PD70 increased *Il6*/*Actb* mRNA in females. There was no statistically detectable difference in males. (F) Relative to SH, SS from PD70 increased *Tlr4*/*ActB* mRNA in females. There was no statistically detectable difference in males. (G-I) Hypothalamic gene expression in animals SH or SS from PD29. (G) Relative to SH, SS from PD29 reduced *Crf*/*Actb* mRNA in females and raised it in males. (H) Relative to SH, SS from PD29 increased *Il6*/*Actb* mRNA in females.cThere was no statistically detectable difference in males. (I) Relative to SH, SS from PD29 increased *Tlr4*/*ActB* mRNA in females. There was no statistically detectable difference in males. *p<0.05, **p<0.01 between housing conditions, †p<0.05, ††p<0.01 between sexes. (from Shannonhouse *et al* 2014a)


A
Corticosterone (ng/ml)
SH
SS
Females
Males
B
Cortisol (ng/ml)
C
Adrenal Weight (mg)
D
Crfl/Actb mRNA (ratio)
E
Il6/Actb mRNA
F
Tlr4/Actb mRNA
G
Crfl/Actb mRNA
SH29
SS29
H
Il6/Actb mRNA
I
Tlr4/Actb mRNA

Effect of dexamethasone phosphate (Dx-p) suppression of endogenous corticosteroids and corticosteroid replacement on anxiety, hedonic drive and gene expression in males

This experiment was an attempt to reconstitute SS female phenotypes in SH males. Animals were given vehicle (water), Dx-p, Dx-p plus cortisol (D+F) or Dx-p plus corticosterone (D+B). Relative to vehicle, D+F animals had increased feed latency and test cage:home cage feed latency ratio in AFEC after 3 days, but no differences were detectable at 10 or 15 days (Fig 2-6A-C). Relative to vehicle, D+B had increased reward investigation in RIP at 10 days and decreased blank investigation at 15 days, but there were no other statistically detectable differences (Fig 2-6D-F). Compared to SH vehicle, SS males, Dx-p and D+B had decreased *Crf* mRNA (Fig 2-6G). There were no statistically detectable differences in *Il6* mRNA, which was probably due to unequal variance (Fig 2-6H). Compared to SH vehicle, Dx-p, D+F and D+B animals had increased *Tlr4* mRNA (Fig 2-6I).

Figure 2-6: **Dexamethasone-phosphate (Dx-p) suppression of adrenal steroids**. SH males (n = 5-8/group) were subjected to no drug (Veh), Dx-p (250µg/kg/day, p.o.), Dx-p + cortisol (D-F; 250µg/kg/day + 50µg/kg/day) or Dx-p + corticosterone (D-B; 250µg/kg/day + 50µg/kg/day) from age 17 weeks. AFEC was assessed on days 3, 10 and 15, RIP was assessed on days 10 and 15 and animals were sacrificed after 7 weeks of treatment and hypothalamic gene expression was assessed (n = 6, 4, 5 and 3 for Veh, Dx-p, D-F and D-B, respectively). (A) Relative to Veh, D-F increased feed latency on day 3. No other effects were statistically detectable. (B) There were no statistically detectable effects on home cage feed latency. (C) Relative to Veh, D-F increased test cage:home cage feed latency ratio on day 3. No other effects were statistically detectable. (D) Relative to Veh, D-B had increased reward investigation time on day 10. No other effects were statistically detectable. (E) Relative to Veh, Dx-p had decreased blank investigation time on day 15. No other effects were statistically detectable. (F) There were no statistically detectable effects on reward preference. (G) Relative to SH, SS, Dx-p and D-B had decreased *Crf*/*Actb* mRNA. (H) There were no statistically detectable effects on *Il6*/*Actb* mRNA. (I) Relative to SH, Dx-p, D-F and D-B had increased *Tlr4*/*ActB* mRNA. *p<0.05, **p<0.01 between Veh and drug or between housing conditions.


A
Feed Latency - Test (s)
Veh
Dex-p
D-F
D-B
Time (days)
B
Feed Latency - Home (s)
Dex-p
D-B
D-F
Veh
Time (days)
C
Feed Latency Ratio
Dex-p
D-B
Veh
D-F
Time (weeks)
D
Reward Investigation (s)
Veh
Dex-p
D-F
D-B
Time (days)
E
Blank Investigation (s)
Dex-p
D-B
D-F
Veh
Time (days)
F
Reward Preference (%)
Dex-p
D-B
Veh
D-F
Time (days)
G
Crfl/Actb mRNA
SH
SS
Dx-p
D-F
D-B
H
Il6/Actb mRNA
SH
SS
Dx-p
D-F
D-B
I
Tlr4/Actb mRNA
SH
SS
Dx-p
D-F
D-B

Discussion

The purpose of these experiments was to establish a sex-biased experimental paradigm linking emotion, metabolism and food intake by a simple manipulation (social housing (SH) or social separation (SS)). Hypophagia is voluntary. Other models manipulate food intake by limiting food available (Ménard *et al* 2014, Kenny *et al* 2014) or increasing calorie intake by using high fat diet (Takase *et al* 2016, Sasake *et al* 2014) or highly palatable food (Rossetti *et al* 2014). Using high fat diet *per se* (Takase *et al* 2016) or uncontrollable stressors (Hartley *et al* 2013, Rozeske *et al* 2012, Sandford *et al* 2010, Cordner *et al* 2004), such as restricting access to food, can affect emotional status. This paradigm allows experiments to measure diet, metabolic and behavior interactions without these confounds.

Depression and anxiety are often comorbid and affect women more often and more severely than men (Grant *et al* 2009). Eating disorders are comorbid with anxiety and depression (Grant *et al* 2009, Hudson *et al* 2007). In humans, loss of social contact is associated with anxiety (Inagaki *et al* 2002, reviewed in Shear and Skritsaya 2012), both anorexia nervosa and anxiety (Sanchez-Cardenas *et al* 1995) and depression (Lee *et al* 2016, reviewed in Kawachi and Berkman 2001). Social isolation is associated with both anxiety and depression, although perceived social isolation is more closely associated with depression than objective social isolation (Chou *et al* 2011, Hawthorne 2008).

This female-biased paradigm linking metabolism, food intake and emotional status may be useful for elucidating mechanisms linking these disorders and this model can help fill that role. Effects of social housing conditions in Syrian hamsters on weight gain, food intake and maintaining juvenile-like linear growth into adulthood have been previously observed (Borer *et al* 1988). Few focused on sex differences (Zhang *et al* 2008, Gatterman *et al* 2002). Both the magnitude and time course of the metabolic effects were female biased. Social separation caused statistically detectable weight loss and hypophagia in females within 1 week and decreased weight gain in males by 4 weeks and hypophagia within 6 weeks.

Increased anxiety-like behavior was statistically detectable in females within 4 weeks of social separation, but increased anxiety-like behavior took 6 weeks in males. Decreased hedonic drive was statistically detectable in females but not in males.

The underlying mechanisms for social separation leading to female-biased hypophagia, elevated metabolic rate, cessation of juvenile-like growth, increased anxiety-like behavior and decreased hedonic drive are unclear. Hamsters fight when socially housed (Goldman and Swanson 1975). Successive social defeat over 4 days increases food intake, body weight and adiposity (Solomon *et al* 2007, Solomon *et al* 2006, Foster *et al* 2005). However, those social encounters were transient and occurred in animals that had been group housed prior to social separation. It is unclear if weight gain, increased food intake and increased adiposity following social defeat are similar to maintaining higher weight gain and food intake with social housing. All socially housed animals, which would include both dominant and subordinates, gained weight (our data, Gattermann *et al* 2002, Fritzsche *et al* 2000, Borer *et al* 1988), while only the subordinates showed increased weight gain in the resident intruder model (Solomon *et al* 2011, Solomon *et al* 2006, Foster *et al* 2005). Corticosteroids alone failed to produce this effect (Solomon *et al* 2011). Experiments presented here focused on the hypothesis that keeping hamsters in social housing into adulthood results in high basal levels of corticosteroids which suppress immune function and reduced corticosteroids after social separation induces inflammation which in turn increases metabolic rate and reduces food intake.

An alternate hypothesis is that early life stress from fighting prior to social separation led to increased anxiety-like behavior, decreased hedonic drive and body weight and food intake phenotypes. Early life stress is associated with increased susceptibility to anxiety and depression in human and animal models (reviewed in Holder and Blaustein 2014) and possibly anorexia and bulimia nervosa (reviewed in Kaye 2008). The data do not support the idea that stress

from fighting is driving anxiety or reduced hedonic drive. Adult fighting begins around 6 weeks (Goldman and Swanson 1975). However, female animals socially separated at 4 weeks (PD29) showed increased anxiety-like behavior in AFEC (Fig 2-4) as well as reduced body weight gain. Males and females had reduced food intake. Therefore, SS had qualitatively similar effects on food intake, metabolism and anxiety-like behavior in juveniles who had never experienced adult fighting compared to adults who had. SS caused a weight gain differences even though animals were rapidly growing juveniles and adolescents. These data suggest effects of SS are at least partly independent of age.

Hypercortisolemia is known to suppress linear growth and bone density in children (Sävendahl 2012) and induce skeletal muscle atrophy (Watson *et al* 2012). During anorexia nervosa, hypercortisolemia is associated with osteoporosis, osteopenia and skeletal muscle atrophy (Fernández-Soto *et al* 2013, McLoughlin *et al* 1998). However, cortisol administration or secretion stimulates growth hormone secretion in humans (Schmid 2008, reviewed in Stratakis 2006). However, SH does not elevate cortisol nor does SS depress cortisol outside the normal physiological range (when cortisol is measured by the same method) (Morgan 2012). Therefore there is no reason to believe changes in corticosteroids within the normal physiological ranges is directly causing metabolic and emotional status phenotypes.

As an alternate test of the adult fighting and/or social contact stress hypothesis, we attempted to suppress HPA axis hormone production with dexamethasone phosphate (Dx-p) in adult, socially housed males in order to reproduce SS behavior phenotypes. SS lowers *Crf* mRNA expression in females and males and raises *Il6* and *Tlr4* mRNA expression in females only. Dx-p suppressed *Crf* to a greater extent than SS and raised *Tlr4* expression. There were no detectable changes in *Il6* expression (possibly due to high variance). Dx-p treatment did not have a detectable effect in AFEC or RIP. Cortisol treatment restored *Crf* expression, but neither cortisol nor corticosterone had a detectable

effect on *Il6* or *Tlr4*. Dx-p HPA suppression did not affect AFEC or RIP, although cortisol and corticosterone replacement increased anxiety-like behavior and reward investigation, respectively. These data provide no evidence that HPA suppression creates SS-like behavior phenotypes.

Another alternate hypothesis is SS during adulthood rather than at weaning was responsible. Hamsters appear to have evolved as solitary animals (Gattermann *et al* 2001). Effects following separation could be developmental artifacts of socially housing hamsters longer than their evolved condition. However, the similar body weight gain and food intake phenotypes as well as anxiety-like behaviors in juvenile (PD29) females following SS argue against this.

In summary, the differences in how males and females respond to social defeat, the effects of SS as juveniles and HPA suppression in males are not consistent with anxiety phenotypes being caused by fighting prior to SS or loss of adolescent-like growth. Effects of territorial aggression, social stress and HPA suppression on hedonic drive are inconclusive.

Further testing is required to rule out or support the hypothesis that inflammation induces hypophagia, increased metabolic rate, decreased weight gain, increased anxiety-like behavior and reduced hedonic drive behavior after SS with a female bias. Inflammation can lower appetite and increase metabolic rate (Langhans 2007, Konsman *et al* 2002, reviewed in Straub 2010) and alter emotional responses (Kullman *et al* 2013, reviewed Salim *et al* 2012). Suppressing endogenous corticosteroids using Dx-p alone was not sufficient to induce SS-like phenotypes in males. However, Dx-p suppression of HPA is not suitable for studying inflammation's effects because Dx-p is anti-inflammatory (reviewed in Wernecke *et al* 2015). *Tlr4* and *Il6* mRNA expression are known markers of neural inflammation (Lu *et al* 2015, Waise *et al* 2015). Relative to SH, both were elevated in SS females but not males. Cortisol is anti-inflammatory (reviewed in Elenkov 2004). Evidence consistent with this hypothesis includes dropping cortisol levels with social separation combined with elevated hypothalamic *Tlr4* and *Il6* mRNA

levels in animals most affected metabolically and emotionally by SS. Other experiments could include inducing inflammation with LPS, suppressing inflammation during SS and monitoring blood markers of inflammation during a SS time course while comparing it to body weight gain, food intake and anxiety-like behavior and hedonic drive behavior time courses.

FLUOXETINE CAUSES PARADOXICAL EFFECTS ON EMOTIONAL STATUS OF ADOLESCENT FEMALE SYRIAN HAMSTERS (*Mesocricetus auratus*) AND ALTERS GAMMA-AMINO BUTYRIC ACID SIGNALING IN THE *NUCLEUS ACCUMBENS**

Summary

Meta-anaylses of clinical trials have found a link between initial treatment of adolescents and young adults with antidepressants worsening on symptoms for anxiety, depression and suicidality. So far, attempts to model paradoxical effects in adolescents with continuous antidepressant treatment have relied on surgically implanting cannulas into the brain for microinjection, surgical implantation of minipumps or drug discontinuation. Consequently, the neurobiological basis for age-dependent differences in responses to chronic orally administered fluoxetine is poorly understood. It is reported here chronic fluoxetine treatment induces increased anxiety-like and depression-like behavior in adolescent, but not adult, Syrian hamster females. The neuroanatomical site of action of these paradoxical effects has not been established, but elevated ionotropic gamma-amino butyric acid (GABA-A) signaling in the nucleus accumbens (NAc) is known to disrupt emotional state. Fluoxetine decreased basal GABA-A signaling in NAc of adults, but increased basal GABA-A signaling in NAc of adolescents. Consistent with the hypothesis GABA-A signaling contributes to fluoxetine's adolescent-specific effects, acute fluoxetine treatment reduced mRNA expression of fluoxetine-insensitive *Gabra5* as well as *Gabra1* and *Gabrd* in adolescents but not adults. The results suggest differences in age-related GABA-A plasticity and GABAergic tone could contribute to paradoxical age differences in emotional status responses to fluoxetine.

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*Part of the data in this section reprinted with permission from Fluoxetine disrupts motivation and GABAergic signaling in adolescent female hamsters. Shannonhouse *et al*. 2016. Progress in Neuropsychopharmacology & Biological Psychiatry. 69:19-30. Copyright 2016 Elsevier BV

Introduction

The United States Food and Drug Administration issued a black box warning on the use of antidepressants in people under the age of 25. Meta-anaylses of clinical trials have found a link between initial treatment of adolescents and young adults with antidepressants worsening on symptoms for anxiety (Bridge *et al* 2007) depression (Bridge *et al* 2007, Cusin *et al* 2007) and suicidality (Stone *et al* 2009, Bridge *et al* 2007, Hammad 2006, Jick *et al* 2004). Antidepressants only show modest therapeutic efficacy in adolescents (Bridge *et al* 2007, Whittington *et al* 2004) or are statistically indistinguishable from placebo in one meta-analysis (Mann *et al* 2006). Fluoxetine has the best risk-benefit profile of common antidepressants in adolescents (Whittington *et al* 2004). There is an urgent need to elucidate the neurological underpinnings for fluoxetine's actions on the adolescent brain.

Due to the limits on human experimentation, an animal model of paradoxical effects of adolescent antidepressant treatment would be useful in understanding mechanisms of these effects and adjusting therapies for younger people. So far, attempts to model paradoxical effects in adolescents with continuous antidepressant treatment have only had success with surgically implanted osmotic minipumps (West at al 2010) or antidepressant discontinuation (Homberg *et al* 2011). Other researchers have failed to produce pro-depressant effects of antidepressants in adolescent rat and mouse models (Karanges *et al* 2011, Iñiguez *et al* 2010, Oh *et al* 2009, de Jong *et al* 2006). In contrast, animal models have had more success in creating anxiogenic responses in adolescents in response to antidepressant treatment (de Jong *et al* 2006, Oh *et al* 2009). However, other published studies have failed to produce similar results (Iñiguez *et al* 2014, Homberg *et al* 2011, Iñiguez *et al* 2010). No published model has shown both paradoxical anxiogenic and pro-depressant effects without the need for surgically implanting cannulas into the brain for microinjection of drugs, surgical implantation of minipumps for drug administration or drug discontinuation. Discontinuation in an animal model calls into question its clinical relevance due to

known, non-paradoxical complications with abrupt antidepressant discontinuation in older adults as well as younger adults and adolescents (reviewed in Schatzberg *et al* 2006, Schatzberg *et al* 1997). The purpose of this section is to show long lasting, robust pro-depressant and anxiogenic effects in adolescent female Syrian hamsters and investigate mechanisms.

While fluoxetine is a selective serotonin reuptake inhibitor (SSRI), it also affects ionotropic γ-amino butyric acid receptor-A (GABA-A) signaling (Ye *et al* 2008, Robinson *et al* 2003). GABA-A signaling is implicated in anxiety (reviewed in Nuss *et al* 2015) and depression (reviewed in Luscher *et al* 2011). Adolescent perturbations of GABA-A signaling are hypothesized to lead to lifelong changes in risk for eating and anxiety disorders (reviewed in Aoki 2016). GABA-A signaling in the nucleus accumbens inhibits hedonic drive (Koo *et al* 2014). GABA-A subunit plasticity alters the sensitivity of GABA-A receptors to drugs during development and is linked to susceptibility to neurological disorders (reviewed in Simeone *et al* 2003) and different subunit expression patterns are associated with paradoxical emotional responses during female endocrine transitions (Backström *et al* 2011, Andreen *et al* 2009, Shen *et al* 2007). Therefore, adolescent-specific effects of fluoxetine on GABA-A signaling in the nucleus accumbens could have a role in mediating fluoxetine's paradoxical effects.

The studies cited above point to 4 major knowledge gaps the experimental paradigm in this section will address. First, the paradigm models worsening of symptoms of anxiety and depression with initial treatment with fluoxetine. Second, the paradigm distinguishes anxiety-like and pro-depressant-like responses of adolescents from adult responses. Third, the paradigm is used to study neurobiological underpinnings of paradoxical responses. Fourth, all studies on paradoxical effects of antidepressants cited above used male animals. A model using female subjects is needed. The paradigm uses adolescent females because they exhibit behaviors that potentially reflect traits for emotional liability (Shannonhouse *et al* 2015, Shannonhouse *et al* 2014a).

This study shows adolescent female hamsters but not adult females have paradoxical increased anxiety-like and decreased hedonic drive responses to fluoxetine. The study focuses on GABA-A signaling and subunit plasticity in the nucleus accumbens as one potential mediator of the paradoxical effects.

Materials and Methods

Animals

Syrian hamsters (*Mesocricetus auratus*) of the Lake View Gorge strain (Charles River, Kingston, NY) were purchased for use at age 8 weeks or were bred in the Kleberg Laboratory Animal Facility at Texas A&M University. Animals were kept on a 14h:10h light:dark schedule (lights on at 0600h) at 23 ± 3˚C. LabDiet 5001 (Purina, Richmond, IN) and water were provided ad libitum. Food intake was monitored by weighing food from the food hopper, the bedding and cheek pouches to the nearest 0.1g. Bedding was Sani-Chips (Murphy's Products, Monteville, NJ). Each cage was supplied with Nestlets (Ancare, Bellmore, NY). One animal was housed per cage starting 2 weeks prior to the experiments. Food was removed from the cages 90 minutes prior to testing. Brains were collected by killing the animals by beheading and freezing in isopentane pre-chilled with dry ice. Procedures used were approved by the Institutional Animal Care and Use Committee.

Anxiety-related feeding/exploration conflict test (AFEC test)

AFEC was performed as described previously (Section 2, Shannonhouse *et al* 2014b). Briefly, an animal is placed in a polycarbonate cage on a white surface in ambient light of ~1000 lux. A plexoglass cover with a hole in the center is placed over the cage to prevent climbing out and graham cracker in a spring loaded clamp is suspended through the hole. Approach and feed latencies were timed.

Reward/investigational preference test (RIP test)

RIP was performed as described previously (Section 2, Shannonhouse *et al* 2015). Briefly, clear plastic cases containing either graham cracker (reward) or nothing (blank) were either placed in the food hopper of a polycarbonate test cage

or suspended through a hole in a clear plastic cover in a spring loaded clamp. Time spent investigating (sniffing, chewing, scratching) each case was measured. Reward investigation time and reward investigational preference (100% * reward investigation time / [reward + blank investigation time]) were used as indices of hedonic drive.

Accumbal microdissection

Frozen brains were blocked, mounted on a freezing microtome stage and sectioned at 150µm. Tissue sections were placed on glass slides on a freezing plate. Using the Hamster Brain Atlas (Morin and Wood 2001) as a guide, ventral striatum containing nucleus accumbens was dissected under a dissecting microscope using 0.75mm metal core punches (Ted Pella, Redding CA).

RNA extraction

RNA was extracted using a modified PIG-B method (Weber *et al* 1998). Briefly, tissues were homogenized in PIG-B, allowed to sit for 15 minutes or more, followed by chloroform addition and centrifugation to separate the aqueous phase. The upper phase underwent two rounds of acidic phenol/chloroform extraction to remove the remaining protein and DNA followed by ethanol precipitation.

Reverse transcription PCR

Reverse transcription was performed using M-MLV reverse transcriptase with random primers (New England Biolabs, Ipswich, MA) according to manufacturer's instructions. PCR reactions were performed using 2X PCR mix (New England Biolabs, Ipswich, MA). Primer sequences are on Table 3-2. All reactions were hot started at 91˚C, had an initial denaturation at 94˚C for 50s. Cycle conditions were melting at temperatures from Table 3-2, hybridization at 60˚C and polymerization at 72˚C for a number of cycles listed on Table 3-2. Bands were visualized on an agarose gel stained with ethidium bromide (ISC Bioexpress, Kaysville, UT) using a FluoroChem HD2 gel doc system (Alpha Innotech, San Leandro, CA) and analyzed band intensity using Kodak 1D Image Analysis Software (Eastman Kodak Co, Rochester, NY). Intensities of genes of interest are

the mean of triplicate or quadruplicate reactions normalized to the median value. Standards were performed in duplicate (Gapdh and Beta Actin primer set 2) or triplicate (Beta Actin primer set 1). All standards were highly correlated with one another (r-square > 0.9 for all pairings). All quantitations were normalized to the mean of the 3 standards.

Acute brain slice preparation

On the day of electrophysiological recordings, coronal slices containing nucleus accumbens were prepared essentially as we have described previously (Dubois *et al* 2004, DuBois *et al* 2006). Hamsters were decapitated on postnatal day 42 or 63. Brains were rapidly excised and cooled by immersion in 0-4°C artificial cerebral spinal fluid (ACSF) containing: 2 mM KCl; 1 mM $MgCl_2$-$6H_2O$; 2 mM $MgSO_4$-$7H_2O$; 1 mM $CaCl_2$; 1.25 mM NaH_2PO_4; 26 mM $NaHCO_3$; 14 mM D-glucose; 206 mM sucrose, 206; 0.8 mM kynurenic acid; bubbled with 95/5% O_2/CO_2; pH 7.4; 290-310 mosM. Brains were blocked and 300-µm slices were cut on a VibroSlice (Campden Instr., Lafayette, IN) in the same solution. Slices were transferred to oxygenated (95/5% O_2/CO_2) ACSF containing: 124 mM NaCl; 3 mM KCl; 1.5 mM $MgSO_4$; 2.4 mM $CaCl_2$; 1.25 mM NaH_2PO_4; 10 mM D-(+)-glucose; 26 mM $NaHCO_3$; pH 7.4; 290-310 mosM. They were gradually warmed to ~32° C, incubated for ~30 min, and then allowed to cool to ~22° C prior to experimentation. Individual slices were transferred to a recording chamber, submerged using a slice anchor (Warner Instr., Hamden, CT) and continuously perfused with the same solution during recordings.

Electrophysiology

Whole-cell patch-clamp techniques were used as described previously (DuBois *et al* 2004, DuBois *et al* 2006). Patch pipettes were pulled from glass capillary tubing (KG-33, 1.5 mm, o.d., Garner Glass, Claremont, CA) on a Brown and Flaming P-97 pipette puller (Sutter Instr., Novato, CA) to resistances of 2-8 MΩ. Pipettes were filled with solution containing: 130 mM CsCl; 10 mM EGTA; 2 mM MgCl2; 10 mM HEPES; 4 mM Mg-ATP; 0.1 mM GTP; pH 7.2 with CsOH;

295–300 mosM. Slices were visualized with Olympus BX50/51 WI upright microscopes (40X long working distance water immersion, differential interference contrast video enhanced optics). Neurons were voltage-clamped at -60 mV and recordings made containing pharmacologically isolated GABA mIPSCs from saline- or fluoxetine-treated hamsters. Tetrodotoxin (0.5 μM, TTX) was used to block Na^+ channels and inhibit action potential-evoked release of neurotransmitter while 40 μM D,L-2-amino-5-phosphonovaleric acid (APV), and 10 μM 6,7-dinitroquinoxaline-2,3-dione (DNQX) were used to inhibit glutamate-mediated mEPSCs. GABA-A-mediated mIPSCs were defined by inhibition with 30 μM bicuculline. The actions of acute fluoxetine application was tested for periods of several minutes with a pair of pipettes (300 μm i.d., glass tubing) under manual control. One pipette delivered bath solution to the recording environment and was displaced by the 2nd pipette for drug application. Drug delivery pipettes were positioned just above the slice over the recorded cell and bath solution or drug was delivered continually over the slice for a sufficient time to allow penetration of drug to the local neuronal environment or drug washout as required. Most drugs reached saturation at the recorded neuron within 2-4 min for cells near the slice surface (i.e., depth of ~ 50-100 μm). Voltage-clamp current recordings were collected and digitized with a Multiclamp 700B amplifier and Digidata 1440A interface and pClamp 10 software (Molecular Devices, Sunnyvale CA). Capacitance (pF) was read from the potentiometer used to zero capacitance transients. Data were low-pass filtered (8 pole Bessel, Frequency Devices) at 1-5 kHz and digitized at 0.5-20 kHz. Series resistance was continually monitored. Data were rejected if resistance increased substantially during the experiments. All experiments were carried out at room temperature.

Miniature postsynaptic current data analysis

Off-line analysis of GABAergic mIPSC kinetic parameters was performed using Minianalysis 6.03 (Synaptosoft, Inc., Decatur, GA), Prism 5 (GraphPad, Inc., San Diego CA), and Microsoft EXCEL as previously described (DuBois et al, 2004, DuBois et al, 2006). The mIPSC characteristics of amplitude, frequency (inter-event interval), and decay time constants were determined and compared. Individual currents ≥15 pA could be clearly distinguished above baseline noise in the 3-4 min current traces collected from individual neurons. Event frequency was determined from the mean inter-event interval, while event peak amplitude was estimated as the absolute difference between the preceding baseline and maxima of the current. For mIPSC decay analysis, low noise traces and non-overlapping events were used to generate an ensemble average mIPSC by aligning the rising phase, and the 10–90% decay phase of this average for each neuron which was fitted with a biexponential function: $y(t) = A_1\exp(-t/\tau_1) + A_2\exp(-t/\tau_2) + A_s$ (1) where A_1 and A_2 are the fraction of the fast and slow decay components, respectively, A_s is the steady-state current, and τ_1 and τ_2 are the fast and slow time constants, respectively. Previously we found that mIPSC ensemble decay data for septal neurons under the similar conditions gave a significantly better fit with two time constants relative to a fit with a single time constant (DuBois et al, 2004).

Drugs

Fluoxetine-HCl (Flu) (Sigma, St. Louis, MO) was dissolved in drinking water at 0.17mg/ml. These concentrations deliver Flu at ~10 mg/kg/day, based on our previous work (Shannonhouse *et al* 2015). To eliminate differences in the onset of initial drug treatment, subjects were injected with Flu (10 mg/kg, ip) dissolved in 0.9% saline at 1200 h on day 1, and behaviors were initially assessed at 1300 h. The drugs were then added to the drinking water at 1800 h on day 1, and replaced every three days. Behaviors of adults were assessed weekly thereafter because fluoxetine was expected to improve performances in the RIP and AFEC tests only after chronic treatment. Because adolescents exhibited markedly increased

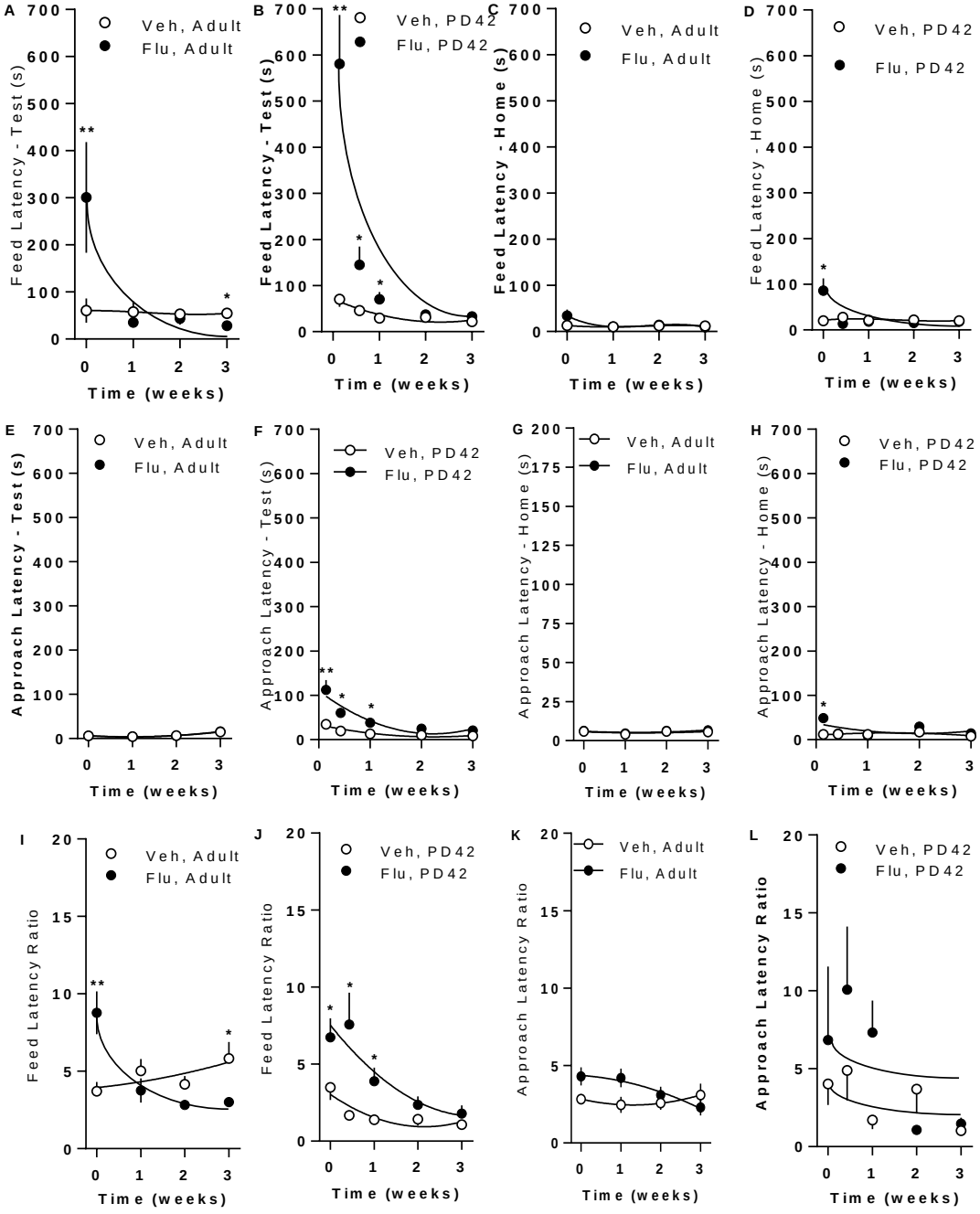
paradoxical behavioral responses to fluoxetine on day1, an additional day of testing was inserted on day 3 for higher resolution analyses. D,L-AP5 and TTX were purchased from Tocris. Kynurenic acid and DNQX were purchased from Sigma.

Results

Effects of acute and chronic fluoxetine treatment on anxiety and hedonic drive by age

This experiment was to determine if adolescent animals responded to fluoxetine in a paradoxical manner in anxiety-related feeding/exploration conflict (AFEC) and reward/investigational preference (RIP) tests and to establish a time course of anxiety-like and hedonic behaviors in adults (postnatal day 63) and adolescents (postnatal day 42) treated with fluoxetine (Flu). Relative to vehicle (Veh), Flu adults show increased feed latency and test cage:home cage feed latency ratio with acute treatment, but they become statistically indistinguishable by day 7 and feed latency and feed latency ratio are decreased by day 21. There are no statistically detectable effects of Flu on home cage feed latency in adults (Fig 3-1A, C, I). In contrast, relative to Veh, Flu adolescent animals show increased feed latency and test cage:home cage feed latency ratio on days 1, 4 and 7, increased home cage feed latency on day 1 and behavior never improves beyond baseline with chronic treatment (Fig 3-1B, D, J). Flu has no statistically detectable effects in adults on approach latency in test cage, home cage or test cage:home cage ratio (Fig 3-1E, G, K). In contrast, relative to Veh, Flu adolescent test cage approach latency and test cage:home cage approach latency ratio were increased for 7 days. Relative to Veh, Flu adolescent home cage approach latency was increased on day 1 (Fig 3-1F, H, L).

Figure 3-1: **Effect of fluoxetine on AFEC in adult and adolescent females**. Females age 9 weeks (adults, PD63) or 6 weeks (adolescents, PD42) were subjected to SS 2 weeks before testing. They received a bolus injection of saline vehicle (Veh) or 10mg/kg fluoxetine (Flu) i.p. on day 1 and water or 10mg/kg/day fluoxetine p.o. in the drinking water. AFEC was tested on days 1, 4, 7, 14 and21. Figure panels are presented as complementary pairs of results in adults and results in adolescents. (A-B) Relative to Veh, Flu increased test cage feed latency on day 1 in adults and days 1-7 in adolescents. Flu decreased feed latency by day 21 in adults. (C-D) Relative to Veh, Flu increased home cage feed latency in adolescents on day 1. (E-F) Relative to Veh, Flu increased test cage approach latency in adolescents on days 1-7. There were no statistically detectable effects in adults. (G-H) Relative to Veh, Flu increased home cage approach latency in adolescents on day 1. (I-J) Relative to Veh, Flu increased test cage:home cage feed latency ratio on day 1 in adults and days 1-7 in adolescents. Flu decreased feed latency ratio by day 21 in adults. (K-L) There were no statistically detectable effects on test cage:home cage approach latency ratio. *$p<0.05$, **$p<0.01$ between Veh and Flu. (from Shannonhouse *et al* 2016)


A
Feed Latency - Test (s)
Veh, Adult
Flu, Adult
Time (weeks)
B
Feed Latency - Test (s)
Veh, PD42
Flu, PD42
Time (weeks)
C
Feed Latency - Home (s)
Veh, Adult
Flu, Adult
Time (weeks)
D
Feed Latency - Home (s)
Veh, PD42
Flu, PD42
Time (weeks)
E
Approach Latency - Test (s)
Veh, Adult
Flu, Adult
Time (weeks)
F
Approach Latency - Test (s)
Veh, PD42
Flu, PD42
Time (weeks)
G
Approach Latency - Home (s)
Veh, Adult
Flu, Adult
Time (weeks)
H
Approach Latency - Home (s)
Veh, PD42
Flu, PD42
Time (weeks)
I
Feed Latency Ratio
Veh, Adult
Flu, Adult
Time (weeks)
J
Feed Latency Ratio
Veh, PD42
Flu, PD42
Time (weeks)
K
Approach Latency Ratio
Veh, Adult
Flu, Adult
Time (weeks)
L
Approach Latency Ratio
Veh, PD42
Flu, PD42
Time (weeks)

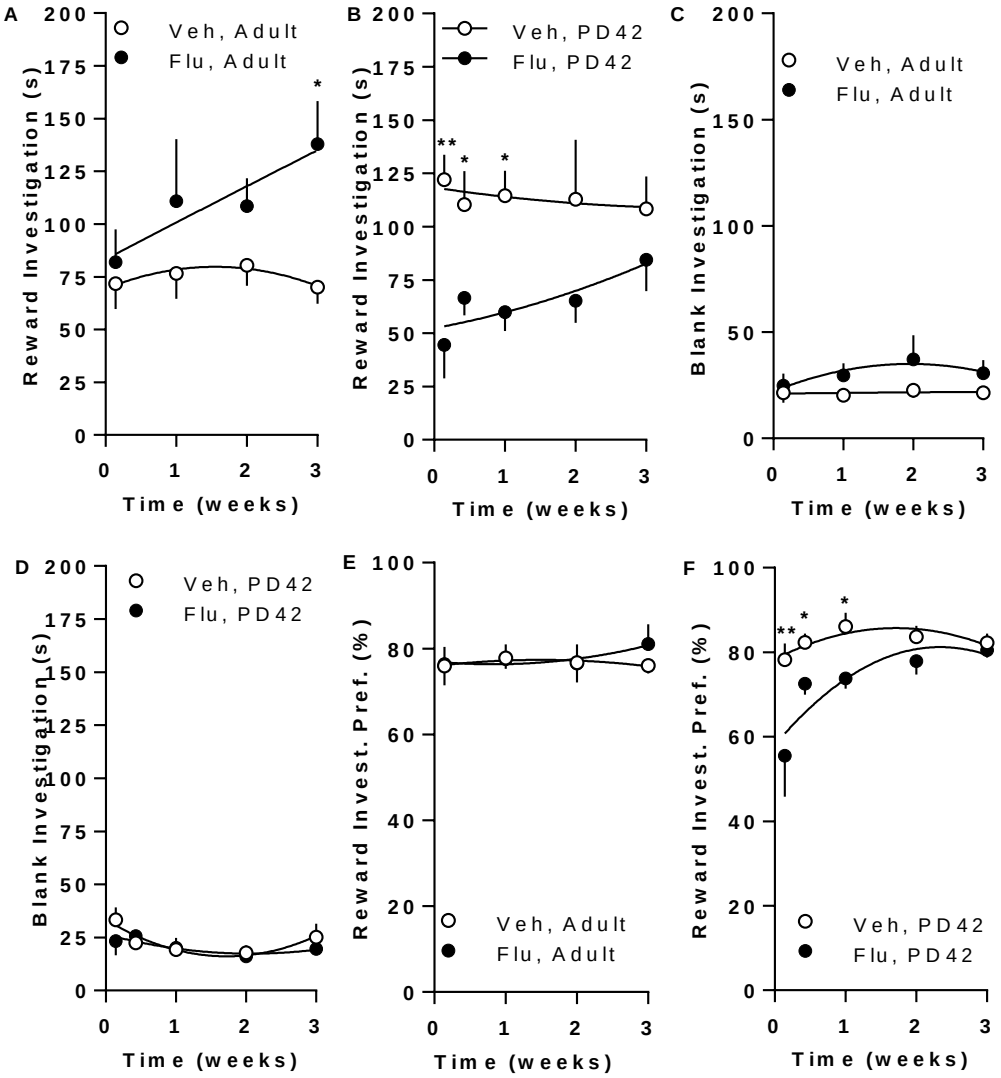


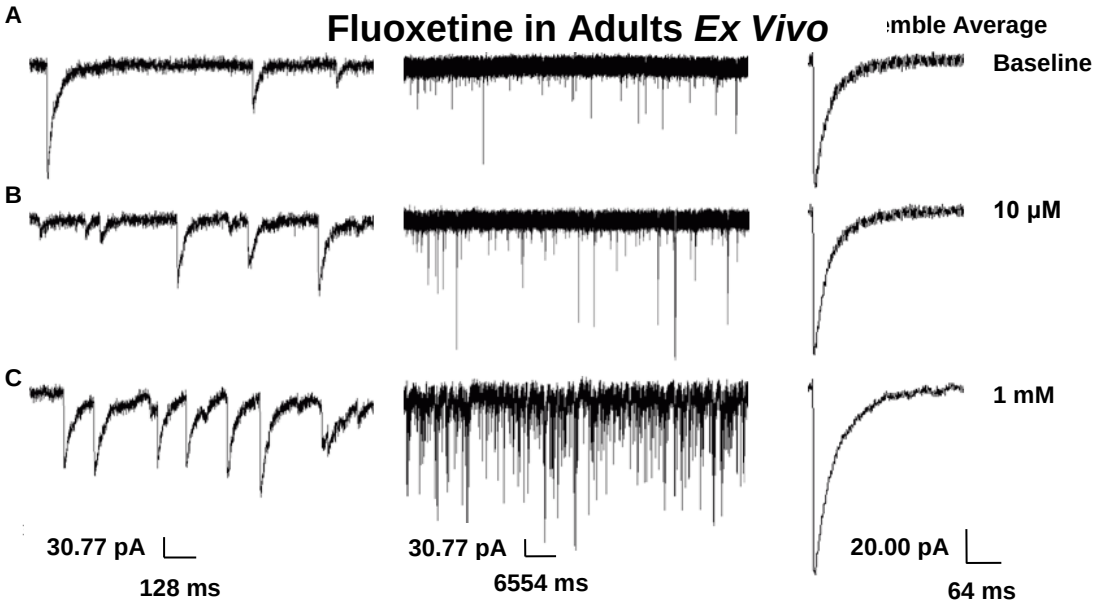
Figure 3-2: **Effect of fluoxetine (Flu) on RIP in adult and adolescent females**. Females age 9 weeks (adults, PD63) or 6 weeks (adolescents, PD42) were subjected to SS 2 weeks before testing. They received a bolus injection of saline vehicle (Veh) or 10mg/kg Flu i.p. on day 1 and water or 10mg/kg/day Flu p.o. in the drinking water. RIP was tested on days 1, 4, 7, 14 and21. Figure panels are presented as complementary pairs of results in adults and results in adolescents. (A-B) Relative to Veh, Flu increased reward investigation in adults by 21 days and decreased reward investigation in adolescents on days 1-7. (C-D) There were no statistically detectable effects on blank investigation. (E-F) Relative to Veh, Flu decreased reward preference on days 1-7 in adolescents. There were no statistically detectable effects in adults. *p<0.05, **p<0.01 between Veh and Flu. (from Shannonhouse *et al* 2016)

RIP was performed on the same animals immediately following AFEC to assess the effects of Flu on hedonic drive. Relative to Veh, Flu adults showed increased reward investigation after 3 weeks. There were no statistically detectable effects on blank investigation or reward preference (Fig 3-2A, C, E). In contrast, Flu in adolescent animals decreased reward investigation and reward preference on days 1, 4 and 7 but had no statistically detectable effect on blank investigation. Reward investigation never improved (Fig 3-2B, D, F).

Effect of acute fluoxetine on GABAergic chloride currents in the nucleus accumbens

The purpose of this experiment was to characterize the effects of acute fluoxetine treatment on GABAergic currents in the *nucleus accumbens*. *Ex vivo* application of fluoxetine causes an immediate, dose-dependent increase in the frequency and amplitude of GABAergic currents in the *nucleus accumbens* (Fig 3-3A-C). Relative to Veh, acute injection of Flu increased both amplitude and frequency of miniature inhibitory post-synaptic currents (mIPSC) in adolescents (Fig 3-3D, E; Fig 3-4A, B). Relative to Veh, Flu decreased mIPSC amplitude in adults, but there was no statistically detectable effect on frequency. The amplitude decrease in adults was concentrated in the highest amplitude mIPSCs (Fig 3-3F, G; Fig 3-4C, D). Relative to adults, adolescents had lower baseline mIPSC amplitude on both Veh and Flu, but the effect size seems to have decreased in the presence of Flu (Fig 3-4E, G). There were no statistically distinguishable differences in mIPSC frequency between adolescents and adults in the presence of Veh or Flu (Fig 3-4F, H).

Figure 3-3: **Representative traces of chloride currents in adolescent (PD42) and adult (PD63) animals with and without fluoxetine (Flu)**. (A-C) Relative to baseline (A), *ex vivo* bath application of Flu increased miniature inhibitory postsynaptic currents (mIPSCs) frequency and amplitude at 10μM and 1mM (B and C, respectively). (D-E) Adolescent and adult animals were injected with saline vehicle (Veh) or 10mg/kg Flu 30 minutes before sacrifice and 2-6 hours later mIPSCs were measured. Relative to Veh, Flu increased mIPSC frequency and amplitude in adolescents. In adults Flu decreased mIPSC amplitude but did not affect frequency. (from Shannonhouse *et al* 2016)


A
Fluoxetine in Adults *Ex Vivo*
mble Average
Baseline
B
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C
1 mM
30.77 pA
128 ms
30.77 pA
6554 ms
20.00 pA
64 ms

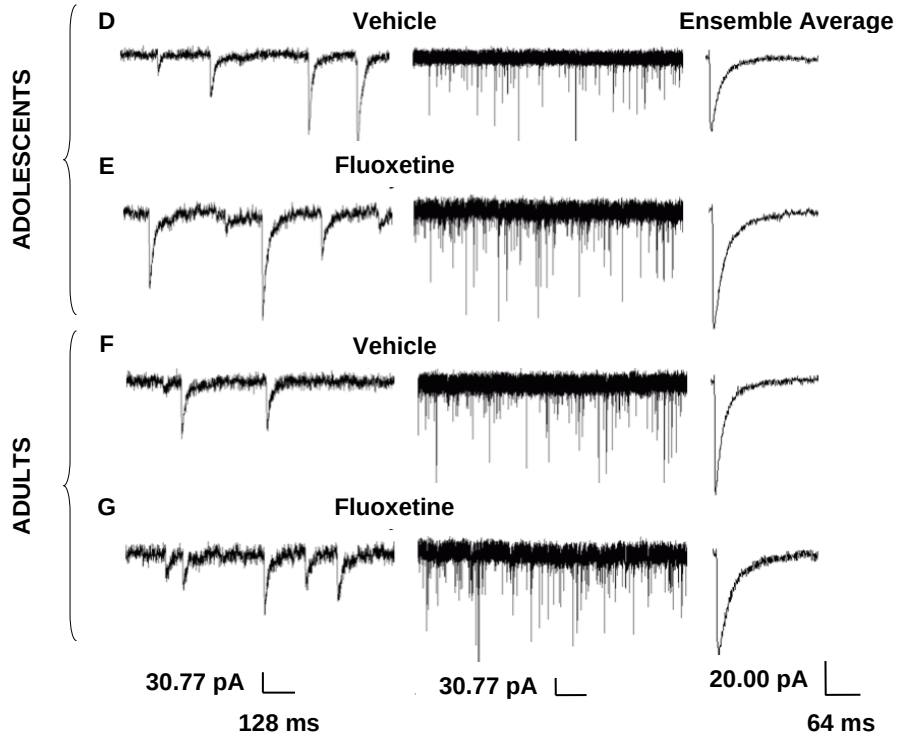
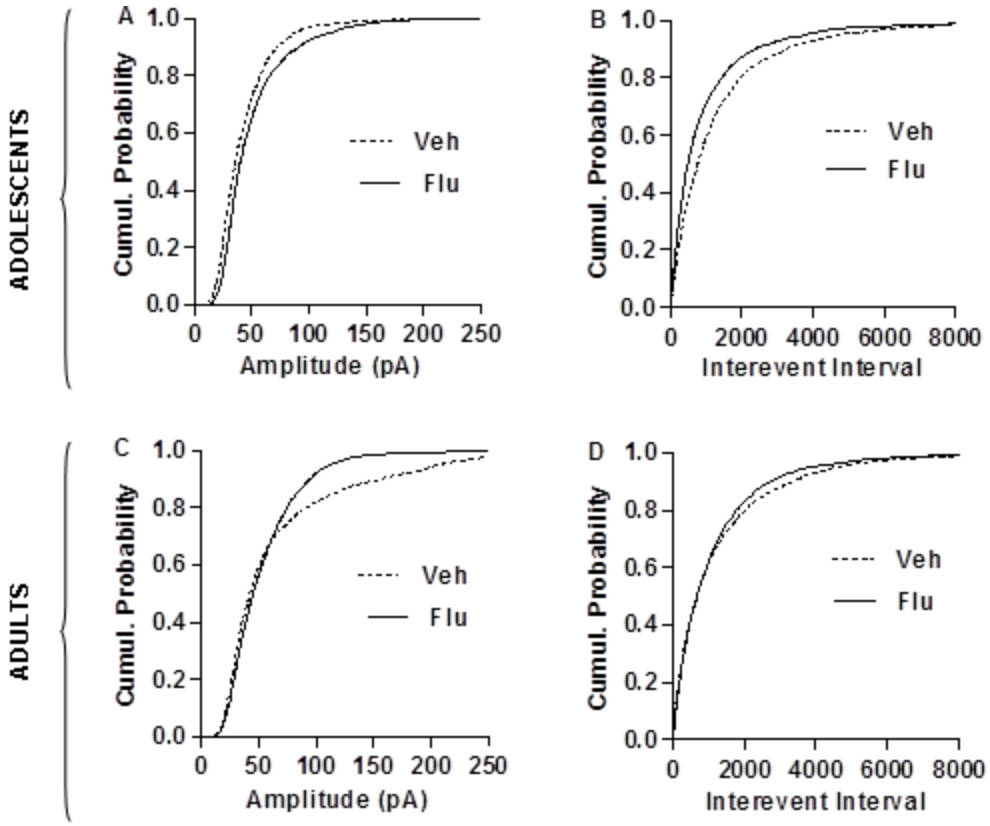

ADOLESCENTS
ADULTS
D
Vehicle
Ensemble Average
E
Fluoxetine
F
Vehicle
G
Fluoxetine
30.77 pA
128 ms
30.77 pA
20.00 pA
64 ms

Figure 3-4: **Cumulative probability plots comparing vehicle v drug within age groups and adult v adolescent within treatment groups**. Adolescent (PD42) and adult (PD63) animals (n = 3/group, 12-16 cells/animal) were injected with saline vehicle (Veh) or 10mg/kg fluoxetine (Flu) 30 minutes before sacrifice and 2-6 hours later mIPSCs were measured. (A) Relative to Veh, Flu increased mIPSC amplitude in PD42 animals. (B) Relative to Veh, Flu increased mIPSC frequency (decreased interevent interval time) in adolescent animals. (C) Relative to Veh, Flu decreased mIPSC amplitude in PD63 animals. (D) There was no statistically detectable effect on mIPSC frequency in adults. (E-F, continued on following page) Relative to PD63 animals, PD42 animals had lower mIPSC amplitude follow *ex vivo* bath application of Flu. There was no statistically detectable difference in mIPSC frequency between PD42 and PD63 animals. (G-H) There were no statistically detectable differences between PD42 and PD63 animals under baseline conditions. (from Shannonhouse *et al* 2016)


ADOLESCENTS
ADULTS
A
B
C
D
Cumul. Probability
1.0
0.8
0.6
0.4
0.2
0.0
0
50
100
150
200
250
Amplitude (pA)
2000
4000
6000
8000
Interevent Interval
Veh
Flu

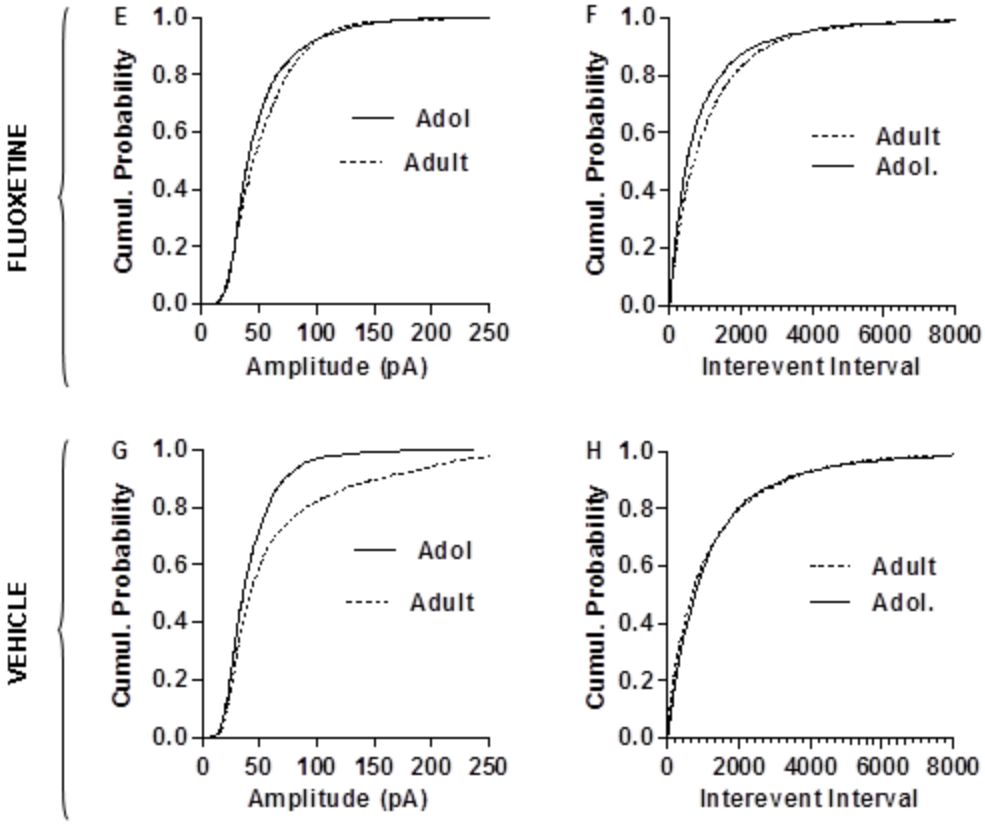


Figure 3-4: **Continued**

Effect of acute fluoxetine on gene expression in the nucleus accumbens of adult and adolescent animals

The purpose of this experiment was to characterize the effects of acute Flu on ionotropic GABA receptor (GABA-A) subunits. Results are summarized on Table 3-1. In general, relative to adults, adolescent GABA-A subunits were less abundant relative to *ActB* and *Gapdh*. This may indicate *ActB* and *Gapdh* are more abundant in adolescent brains than adult. Several subunits broke this pattern: *Gabra3*, *Gabra5* and *Gabra6* were the same abundance in adolescents as in adults which means they were increased relative to other GABA-A subunits. Relative to Veh, *Gabra1*, *Gabra2* and *Gabra5* all showed a main effect of Flu treatment in 2-way ANOVA. *Gabra1* and *Gabra5* were decreased in adolescents in post-hoc t-tests and *Gabra2* was decreased in adults. Relative to Veh, *Gabrb2* and *Gabrd* were decreased in Flu adults and adolescents, respectively, despite not showing a main drug effect in 2-way ANOVA.

The experiment also looked at markers of anxiety, hedonic drive and the serotonin system (results summarized in Table 3-1). As with GABA-A subunits, relative to adults markers of emotional behavior (*Bdnf*, *Creb1*, *FosB* and *ΔFosB*) were all lower relative to *ActB* and *Gapdh* in adolescents. Relative to Veh, *FosB* was decreased in Flu adults. Relative to Veh, *Bdnf*, *Creb1* and *FosB* were decreased in Flu adolescents, although *Bdnf* did not show a main effect of drug in 2-way ANOVA. Relative to Veh, was decreased as a main effect in 2-way ANOVA in Flu animals, but no post-hoc t-tests showed a significant difference. Relative to Veh, *5Ht1a* was decreased as a main effect of Flu in 2-way ANOVA, although post-hoc t-tests showed no difference. Notably, *5Ht1a* was undetectable in most Flu adolescents. Relative to Veh, *Tph1* increased in Flu adults but decreased in Flu adolescents. Relative to Veh adults, *Tph1* was increased in Veh adolescents. However, relative to Flu adults *Tph1* was decreased in Flu adolescents.

Table 3-1: **Ventral striatum gene expression in adults and adolescents with vehicle or fluoxetine**. Adult (postnatal day 63, PD63) or adolescent (PD42) female hamsters were injected with saline vehicle or 10mg/kg fluoxetine, i.p. and sacrificed 3 hours later. Gene expression was measured by PCR normalized to the mean of 2 different *Actb* amplicons and *Gapdh* (n = 6, 6, 5 and 4 for PD63 Veh, PD63 Flu, PD42 Veh and PD42 Flu, respectively). Statistically detectable differences by drug treatment are highlighted in **bold type** and differences by age are highlighted with *italics* and gray shading. Differences highlighted in columns 2-5 (adult and adolescent, Veh and Flu) indicate differences by post-hoc t-tests (Adult Veh v Adolescent Veh, Adult Flu v Adolescent Flu, Adult Veh v Adult Flu or Adolescent Veh v Adolescent Flu). *p < 0.05 and **p < 0.01 by fluoxetine treatment or fluoxetine x age interaction. †p < 0.05 and ††p < 0.01 by age. (partly from Shannonhouse *et al* 2016)

Table 3-1 Ventral striatum gene expression in adults and adolescents with vehicle or fluoxetine							
mRNA	Adult, Veh	Adult, Flu	Adolescent, Veh	Adolescent, Flu	F, age	F, drug	F, interact.
Gabra1	1.737 ± 0.226	1.356 ± 0.167	*0.693 ± 0.102††*	***0.353 ± 0.029*††***	*$F_{1,18}$ = 39.7††*	**$F_{1,18}$ = 4.92***	$F_{1,18}$ = 0.0159
Gabra2	1.538 ± 0.080	**1.201 ± 0.090****	*0.815 ± 0.052††*	*0.605 ± 0.060††*	*$F_{1,17}$ = 12.2††*	**$F_{1,17}$ = 70.9****	$F_{1,17}$ = 0.658
Gabra3	1.72 ± 0.21	1.80 ± 0.20	1.72 ± 0.13	1.80 ± 0.20	$F_{1,18}$ = 0.011	$F_{1,18}$ = 0.011	$F_{1,18}$ = 0.097
Gabra4	2.44 ± 0.26	2.07 ± 0.18	1.81 ± 0.28	1.66 ± 0.10	*$F_{1,18}$ = 5.568†*	$F_{1,18}$ = 1.392	$F_{1,18}$ = 0.249
Gabra5	1.079 ± 0.154	0.881 ± 0.107	1.109 ± 0.067	**0.839 ± 0.081***	$F_{1,17}$ = 0.161	**$F_{1,17}$ = 7.113***	$F_{1,17}$ = 0.014
Gabra6	1.079 ± 0.154	0.881 ± 0.107	1.109 ± 0.067	0.839 ± 0.081	$F_{1,17}$ = 1.705	$F_{1,17}$ = 1.067	$F_{1,17}$ = 0.064
Gabrb1	1.433 ± 0.114	1.282 ± 0.122	*0.697 ± 0.095††*	*0.679 ± 0.066††*	*$F_{1,17}$ = 36.39††*	$F_{1,17}$ = 0.580	$F_{1,17}$ = 0.359
Gabrb2	1.46 ± 0.139	**1.11 ± 0.067***	*0.823 ± 0.066††*	*0.734 ± 0.066†*	*$F_{1,17}$ = 22.59††*	$F_{1,17}$ = 4.242	$F_{1,17}$ = 1.499
Gabrg1	1.689 ± 0.345	1.194 ± 0.152	*0.730 ± 0.080†*	*0.628 ± 0.100†*	*$F_{1,17}$ = 11.19††*	$F_{1,17}$ = 1.714	$F_{1,17}$ = 0.743
Gabrg2	1.657 ± 0.259	1.113 ± 0.125	*0.778 ± 0.047†*	0.758 ± 0.086	*$F_{1,17}$ = 12.64††*	$F_{1,17}$ = 2.641	$F_{1,17}$ = 2.280
Gabrd	1.847 ± 0.281	1.352 ± 0.181	*0.709 ± 0.066††*	***0.450 ± 0.040**††***	*$F_{1,18}$ = 5.675††*	$F_{1,18}$ = 0.775	$F_{1,18}$ = 0.076
Gad67	1.655 ± 0.326	1.247 ± 0.218	*0.608 ± 0.054††*	*0.628 ± 0.042††*	*$F_{1,18}$ = 14.47††*	$F_{1,18}$ = 0.785	$F_{1,18}$ = 0.955
Bdnf	1.148 ± 0.206	1.207 ± 0.115	0.977 ± 0.075	***0.677 ± 0.072*††***	*$F_{1,18}$ = 6.395†*	$F_{1,18}$ = 0.756	$F_{1,18}$ = 1.677
Creb1	2.187 ± 0.307	1.133 ± 0.180	*0.846 ± 0.044††*	***0.687 ± 0.070**††***	*$F_{1,15}$ = 21.42††*	**$F_{1,15}$ = 9.871****	***$F_{1,15}$ = 5.374****
ΔFosB	1.206 ± 0.135	1.017 ± 0.097	0.804 ± 0.153	0.454 ± 0.036	*$F_{1,15}$ = 15.86††*	**$F_{1,15}$ = 4.947***	$F_{1,15}$ = 0.441
FosB	1.532 ± 0.122	**0.960 ± 0.144****	*0.770 ± 0.076††*	***0.487 ± 0.040*††***	*$F_{1,15}$ = 31.77††*	**$F_{1,15}$ = 15.23****	$F_{1,15}$ = 1.740
5HT1A	1.450 ± 0.319	1.235 ± 0.117	0.736 ± 0.213	0.006 ± 0.006	*$F_{1,18}$ = 122.1††*	**$F_{1,18}$ = 28.88****	***$F_{1,18}$ = 8.579****
Tph1	0.755 ± 0.110	**1.226 ± 0.123****	*1.355 ± 0.187††*	***0.622 ± 0.151*††***	$F_{1,18}$ = .001	**$F_{1,18}$ = 4.588***	***$F_{1,18}$ = 96.89****

Discussion

Fluoxetine has paradoxical long-lasting anxiogenic and reduced hedonic drive effects in adolescent female hamsters, but not adult females or adolescent or adult males. No stress, injury or discontinuation was required to produce these effects. This model produced long lasting differences between adult and adolescent females treated with fluoxetine. Both had increased feed latency relative to no-drug controls on day 1 which is unsurprising because SSRI's are often acutely anxiogenic in rodents (Arrant *et al* 2013, Liu *et al* 2010, Griebel *et al* 1995, Kshama *et al* 1990, reviewed in Spigset 1999). However, increased feed latency and feed latency ratio persisted and reduced reward investigation persisted seven days in fluoxetine treated adolescents. In adults, extended fluoxetine treatment increased reward investigation and decreased feed latency relative to controls by 3 weeks. This model of paradoxical effects has good face validity to study the paradoxical effects of antidepressants in adolescents that led to the FDA's black box warning for prescribing antidepressants to patients younger than 25.

Fluoxetine is anorexogenic (Wellman *et al* 2003). However, decreased feed latency in AFEC and reduced food investigation in RIP were not caused by reduced appetitive or consummatory drive. AFEC does not measure appetitive drive in hamsters (Section 2, Shannonhouse *et al* 2014a). Home cage feed and approach latencies were unaffected by day 4 in any treatment group, arguing against an anorexigenic effect (Fig 3-1). Adult hamsters showed decreased feed latency and increased hedonic drive over time despite fluoxetine's anorexogenic effects (Fig 3-1, 3-2). Therefore, our results are not consistent with the anorexogenic effects being a major contributor to the anxiogenic and reduced hedonic drive effects of fluoxetine in adolescents.

Other models have had limited success in producing adolescent-specific, paradoxical effects with antidepressants. Continuous infusion of paroxetine by osmotic minipump produced depression-related behaviors in male adolescent rats (West *et al* 2010), but similar fluoxetine experiments only produced anxiogenic

responses in adolescent male mice without pro-depressant responses (Oh *et al* 2009). Drinking water administration of paroxetine to adolescent male rats produced anxiogenic but not pro-depressant responses (Karranges *et al* 2011). Other experiments used discontinuation to produce adolescent-specific depression-like behaviors in male rats (Homberg *et al* 2011) or anxiety-like behaviors in male rats and mice, respectively (de Jong *et al* 2006, Oh *et al* 2009). However, other discontinuation experiments failed to produce depression-like behavior (Iñiguez *et al* 2014, Iñiguez *et al* 2010) or anxiety-like behavior (Homberg *et al* 2011). This model produces anxiety- and depression-like behaviors without needing surgically implanted cannulas for microinjection into the brain, surgically implanted minipumps or discontinuation of drugs. Therefore, this model has better face validity than rat or mouse models because it better resembles drug administration in humans, is more convenient, technically easier and cheaper to perform than rat or mouse models and does not have the confound of surgical stress.

Several lines of evidence support the importance of GABAergic tone in depression and anxiety. GABA concentrations are reduced in the cerebral cortex and cerebrospinal fluid of depressed humans (Honig *et al* 1988, Kasa *et al* 1982). Selective serotonin reuptake inhibitors including fluoxetine restore GABA to normal levels in the occipital cortex of depressed humans (Honig *et al* 1988) and increase GABA in the CSF of rats (Gören 2007). Chronic mild unpredictable stress lowers GABA concentrations in rat hippocampus (Grønli *et al* 2007). At least one GABA mimetic drug has been shown to have antidepressant effects (Magni *et al* 1989). Anxiolytic drugs often target ionotropic GABA receptors (reviewed in Nuss 2015). Fluoxetine is a known potentiator of ionotropic GABA receptor alpha subunits including α1, α2, α3, α4 and α6 but not α5 (Ye *et al* 2008, Robinson *et al* 2003). These results suggest that, compared to adult females, adolescent females have lower basal GABAergic tone in based on mIPSCs.

Fluoxetine treatment 30 minutes before a kill decreased amplitude of GABA mIPSCs in the Nucleus accumbens (NAc) in adult females, but increased amplitude in adolescent females. Fluoxetine produced no statistically detectable effect of mIPSC frequency in adults, but increased the frequency of mIPSCs in adolescents. This suggests and overall decrease in basal GABAergic tone in adults and a large increase in GABAergic tone in adolescents.

mRNA expression of GABA receptor subunits indicated either an overall decrease in the amount of GABA receptor mRNA in the Nucleus accumbens and/or higher Actb and Gapdh mRNA. Notably, fluoxetine-insensitive α5 was comparable in adolescents, unaffected by fluoxetine treatment by post-hoc t-test in adults and reduced in abundance by fluoxetine treatment in adolescents. Down regulating the fluoxetine insensitive subunit could make the animals even more sensitive to fluoxetine's effects on the GABA system.

A hypothesis about how fluoxetine affects adolescents differently via ionotropic GABA receptors is high basal GABAergic tone in the NAc decreases hedonic drive in adolescents but not adults. Note that this hypothesis is not mutually exclusive with an effect on serotonin receptor signaling. Published experiments suggest direct GABA-A effects are not good candidates for fluoxetine's paradoxical effects on anxiety. GABA-A agonist microinjections into the Nucleus accumbens causes anxiolytic effects in rats (Lopes *et al* 2007). Lowered GABA-A expression and activity in the NAc is associated with reduced morphine reward and GABA-A antagonists increase morphine reward (Koo *et al* 2014). GABA-A antagonists in NAc decrease NAc dopamine release (Rahmen *et al* 2002, Schulte *et al* 2000). NAc infusion of D1 and D2 dopamine receptor agonists are anxiolytic (Ahmadi *et al* 2013). Therefore, a direct effect on GABA-A is a good candidate for paradoxical effects on fluoxetine on hedonic drive, while anxiogenic effects of fluoxetine mediated by GABA-A in NAc would have to be indirect. Further tests of this hypothesis could be using ionotropic GABA agonists to decrease hedonic drive in adolescent but not adult females, using GABA-A

partial agonists or antagonists to block fluoxetine's paradoxical effects in adolescents and measuring GABA-A expression and/or electrophysiology in animals after testing to see if adolescent but not adult females showing low hedonic drive are the same ones with high GABA-A activity and have the strongest paradoxical effects.

CONCLUSIONS

The purpose of these studies was to use Syrian hamsters (*Mesocricetus auratus*) to address experimental problems that have been difficult to address in mice and rats. Two novel tests, the feeding/exploration conflict (AFEC) test for anxiety-like behavior and reward investigational preference (RIP) test for hedonic drive/depression-like behavior, are low stress and low labor intensity methods which can be performed together and respond as predicted to pharmacological treatments. They remain sensitive to pharmacological treatments after repeated tests. Both tests distinguish appetitive behavior from emotional behavior by preventing cheek pouching. The social housing/social separation (SH/SS) experimental paradigm links emotional status, metabolism and hypothalamic-pituitary-adrenal axis activity with female sex bias. SH/SS experiments may help elucidate links between social interaction, emotional status and metabolism and elucidate neurological underpinnings of sex biases in anxiety, depression and metabolic states. Finally, treatment of adolescent female hamsters with fluoxetine represents the easiest experimental approach to producing paradoxical effects of antidepressants in adolescent but not adult animals. With no need for surgical implants for drug administration or discontinuation, adolescent female hamsters show the best face validity of any experimental system for studying paradoxical effects of antidepressants.

Low hedonic drive is a component of depression (Sharpley and Bitsika 2013). Progressive ratio is a food reward based test to measure hedonic drive (Leventopoulos *et al* 2009, Hodos 1961, reviewed in Stafford *et al* 1998) that has a potentially long duration of a test session, may be unreliable in measuring effects of drugs or stressors with short duration effects and subjects becoming sated with or developing tolerance to a reward (reviewed in Stafford *et al* 1998). Sucrose preference is another test for hedonic drive (Kubera *et al* 2013, Muscat *et al* 1992, Willner *et al* 1987). A disadvantage of sucrose preference is the requirement for a schedule of stress which makes the process both labor-intensive and difficult to

perform multiple times on the same animal (Strekalova *et al* 2011). The relative ease and low stress of RIP makes it easier and cheaper to perform large experiments and follow the effects of a treatment over time. RIP can be performed immediately after AFEC. Since anxiety and depression are often comorbid (Grant *et al* 2009), AFEC and RIP make an appealing combination.

There are several advantages to the SH/SS experimental paradigm linking emotion, metabolism and food. Hypophagia is voluntary. Other models manipulate food intake by limiting food available (Ménard *et al* 2014, Kenny *et al* 2014), increasing calorie intake by using high fat diet (Takase *et al* 2016, Sasake *et al* 2014) or highly palatable food (Rossetti *et al* 2014). Using high fat diet *per se* (Takase *et al* 2016) or uncontrollable stressors (Hartley *et al* 2013, Rozeske *et al* 2012, Sandford *et al* 2010, Cordner *et al* 2004), such as restricting access to food, can affect emotional status. This paradigm allows experiments to measure diet, metabolic and behavior interactions without these confounds. Another advantage of the SH/SS paradigm is sex bias giving it face validity for modeling depression and anxiety in humans. Depression and anxiety are affect women more often and more severely than men (Grant *et al* 2009).

The SH/SS paradigm may be useful in elucidating the neurological underpinnings of links between eating disorders and social interaction and emotional disorders. Eating disorders are comorbid with anxiety and depression (Grant *et al* 2009, Hudson *et al* 2007). In humans, loss of social contact is associated with anxiety (Inagaki *et al* 2002, reviewed in Shear and Skritsaya 2012), both anorexia nervosa and anxiety (Sanchez-Cardenas *et al* 1995) and depression (Lee *et al* 2016, reviewed in Kawachi and Berkman 2001). Social isolation is associated with both anxiety and depression, although perceived social isolation is more closely associated with depression than objective social isolation (Chou *et al* 2011, Hawthorne 2008).

Mouse and rat models have had limited success in producing adolescent-specific, paradoxical effects with antidepressants (Homberg *et al* 2011, Karranges

et al 2011, West *et al* 2010, Oh *et al* 2009, de Jong *et al* 2006). Fluoxetine has paradoxical long-lasting anxiogenic and reduced hedonic drive effects in adolescent female hamsters, but not adult females or adolescent or adult males. This model does not rely on surgical implants, stress or discontinuation to produce these effects. Consequently, the model has better face validity to elucidate the neurological underpinnings of paradoxical effects of antidepressants in adolescents.

The results of these experiments suggest that, compared to adult females, adolescent females have lower basal GABAergic tone in based on mIPSCs. Several lines of evidence support the importance of GABAergic tone in depression and anxiety (Gören 2007, Grønli *et al* 2007, Magni *et al* 1989, Honig *et al* 1988, Kasa *et al* 1982). Anxiolytic drugs often target ionotropic GABA receptors (reviewed in Nuss 2015). Fluoxetine is a known potentiator of ionotropic GABA receptor alpha subunits including α1, α2, α3, α4 and α6 but not α5 (Ye *et al* 2008, Robinson *et al* 2003).

A hypothesis about how fluoxetine affects adolescents differently via ionotropic GABA receptors is high basal GABAergic tone in the NAc decreases hedonic drive in adolescents but not adults. Decreased GABA-A and increased activity in the NAc are associated with reduced and increased morphine reward, respectively (Koo *et al* 2014). GABA-A antagonists in NAc decrease NAc dopamine release (Rahmen *et al* 2002, Schulte *et al* 2000). Therefore, a direct effect on GABA-A is a good candidate for paradoxical effects on fluoxetine on hedonic drive

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et al 2013). Anxiogenic effects of fluoxetine mediated by GABA-A in NAc would have to be indirect.

Syrian hamsters models can help elucidate neurobiological underpinnings of links between emotional status and metabolism as well as paradoxical effects of antidepressants in adolescents. Modeling anxiety and depression can be helped with novel low stress, low labor intensity tests that remain sensitive to anxiolytics and antidepressants with repetition.

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APPENDIX

Table A-1: PCR primers and conditions for SH/SS animal experiments		
Gene:	Primer Sequence	Conditions
Actb	Fwd: GGTATGGAATCCTGTGGCATCCATGA Rev: ACTCCTGCTTGCTGATCCACATCT	
Tlr4	Fwd: CTCCCTGAGACCTGAAAGCTTGGAT Rev: GGTGTAGACCCTGATATGCCTTGTCTT	11 *Tlr4* 26 *Tlr4* + *Actb*
Il6	Fwd: ACTTCTCAACAAGTCGGAGGTTTGGTTA Rev: CATCAGGATGGCCTTGGAGGTT	15 *Il6* 26 *Il6* + *Actb*
Crf	Fwd: CGCTAACTTTTTCCGCGTGTTGCT Rev: TGAGCTTGCTGTGCTAACTGCTCT	11 *Crf* 26 *Crf* + *Actb*

Table A-1: **PCR primers and conditions for SH/SS animal experiments**. PCR was performed by a pre-amplification of low abundance transcripts followed by adding primers for the *Actb* standard. (from Shannonhouse *et al* 2014a)

Table A-2: **PCR primers and conditions for Flu treatment experiments**. Temperatures of hybridization and polymerization and times spent at each temperature were constant. (from Shannonhouse *et al* 2016)

Table A-2: PCR primers and conditions for Flu treatment experiments		
Gene:	Primer Sequence	Conditions
Beta Actin (set 1)	Fwd: TCGTACCACAGGCATTGTGATGGA Rev: TGGCCATCTCCTGCTCGAAGT	22 cycles, T_M 93°C
Beta Actin (set 2)	Fwd: GGTATGGAATCCTGTGGCATCCATGA Rev: ACTCCTGCTTGCTGATCCACATCT	22 cycles, T_M 93°C
Gapdh	Fwd: GGCAAGGTCATCCCAGAGCTGAA Rev: GCCTGCTTCACCACCTTCTTGATGT	23 cycles, T_M 91°C
Gaba -A α1 receptor	Fwd: TAGACCAGGACTGGGAGACAGTA Rev: CCATATCTGTATCTGAGACAGGGCCAAA	24 cycles, T_M 91°C
Gaba -A α2 receptor	Fwd: TGCACCTGATGGGTCCAGGTT Rev: TCATGATGCAAGGCAGATAGGTCTGAA	28 cycles, T_M 91°C
Gaba -A α3 receptor	Fwd: TGGACTGGTTCATAGCCGTCTGTT Rev: CCAAGCCCAACTTCGCTTAGTGA	29 cycles, T_M 91°C
Gaba -A α4 receptor	Fwd: TTTGGACCCCTGATACTTTCTTCAGGA Rev: CCGCACTTATGGTGAGTCTCATTGTG	26 cycles, T_M 94°C
Gaba -A α5 receptor	Fwd: TGACTGCCCATTTCCACCTGAAGA Rev: GGTCAGCACTGTGGTCACTCCAA	28 cycles, T_M 91°C
Gaba -A α6 receptor	Fwd: GGACGGACATGCTTGTCCACTCA Rev: TCATACTGGAGGAGGCTGGAAGA	34 cycles, T_M 91°C
Gaba -A β1 receptor	Fwd: GCATCCTGATGGAACCGTCCTCTA Rev: TGCTCCCTCTCCTCCATTCCA	29 cycles, T_M 91°C
Gaba -A β2 receptor	Fwd: CCTAAGGCGGTATCCACTGGATGA Rev: ACGATGGAGAACTGAGGAAGCTCAA	27 cycles, T_M 91°C
Gaba -A γ1 receptor	Fwd: CCCTCTGTGGAAGTAGCTGATCCTAA Rev: CCCATCCGTCTGCTCAGGTCAA	35 cycles, T_M 91°C
Gaba -A γ2 receptor	Fwd: CGACACGAGATCTTGGAGGCTGTA Rev: AGGACACCCAGGATAGAACCACGAT	35 cycles, T_M 91°C
Gaba-A δ receptor	Fwd: GGGACAGCAGGCTCTCTTATAACCAT Rev: CGTGGAGGTGATGCGGATGCTA	32 cycles, T_M 91°C
Gad67	Fwd: GGAGCAGATCCTGGTTGACTGTAGAGA Rev: TCCATGAGAACAAACACGGGTGCAA	26 cycles, T_M 91°C
Npas4	Fwd: CCTTCTAGGCCTGAGCCTTCTCT Rev: GGTGCTTGGTGTCAGCTGTTCT	28 cycles, T_M 91°C
Trph	Fwd: CCAGGAGAACCACGTGAACCTGTTA Rev: AGAGCATAGTGGTGTGGGACTTCA	31 cycles, T_M 91°C
5Ht1A	Fwd: TGCTCTGTACCAGGTGCTCAACAA Rev: GCCAATGAGCCAAGTGAGCGAGAT	29 cycles, T_M 91°C
ΔFosB	Fwd: GGAGGGTTCGCAGAGAGAGAAACAA Rev: CCGAGGACTTGAACTTCACTCGG	31 cycles, T_M 94°C
FosB	Fwd: GGAGGGTTCGCAGAGAGAGAAACAA Rev: CGAGGACTTGAACTTCACTGTGTGT	31 cycles, T_M 94°C
Creb	Fwd: ACAGATTGCCACATTAGNCCAGGTA Rev: TCCACAGACTCCTGTGAATCTTCACT	28 cycles, T_M 92°C
Bdnf	Fwd: ATGGGACTCTGGAGAGCGTGAAT Rev: AGGAGGCTCCAAAGGCACTTGA	28 cycles, T_M 91°C